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(54) Title: NF-AT POLYPEPTIDES AND POLYNUCLEOTIDES (57) Abstract The invention provides novel polypeptides which are associated with the transcription complex NF-AT, polynucleotides encoding such polypeptides, antibodies which are reactive with such polypeptides, polynucleotide hybridization probes and PCR amplification probes for detecting polynucleotides which encode such polypeptides, transgenes which encode such polypeptides, homologous targeting constructs that encode such polypeptides and/or homologously integrate in or near endogenous genes encoding such polypeptides, nonhuman transgenic animals which comprise functionally disrupted endogenous genes that normally encode such polypeptides, and transgenic nonhuman animals which comprise transgenes encoding such polypeptides. The invention also provides methods for detecting T cells (including activated T cells) in a cellular sample, methods for treating hyperactive or hypoactive T cell conditions, methods for screening for immunomodulatory agents, methods for diagnostic staging of lymphocyte differentiation, methods for producing NF-AT proteins for use as research or diagnostic reagents, methods for producing antibodies reactive with the novel polypeptides, and methods for producing transgenic nonhuman animals.		

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NF-AT POLYPEPTIDES AND POLYNUCLEOTIDESFIELD OF THE INVENTION

5 The invention provides novel polypeptides which are associated with the transcription complex NF-AT, polynucleotides encoding such polypeptides, antibodies which are reactive with such polypeptides, polynucleotide hybridization probes and PCR amplification probes for detecting
10 polynucleotides which encode such polypeptides, transgenes which encode such polypeptides, homologous targeting constructs that encode such polypeptides and/or homologously integrate in or near endogenous genes encoding such polypeptides, nonhuman transgenic animals which comprise functionally disrupted
15 endogenous genes that normally encode such polypeptides, and transgenic nonhuman animals which comprise transgenes encoding such polypeptides. The invention also provides methods for detecting T cells (including activated T cells) in a cellular sample, methods for treating hyperactive or hypoactive T cell
20 conditions, methods for screening for immunomodulatory agents, methods for diagnostic staging of lymphocyte differentiation, methods for producing NF-AT proteins for use as research or diagnostic reagents, methods for producing antibodies reactive with the novel polypeptides, and methods for producing
25 transgenic nonhuman animals.

BACKGROUND OF THE INVENTION

 The immune response is coordinated by the actions of cytokines produced from activated T lymphocytes. The
30 precursors for most T lymphocytes arise in the bone marrow and migrate to the thymus where they differentiate and express receptors capable of interacting with antigen. These differentiated T lymphocytes then migrate to the peripheral lymphoid organs where they remain quiescent until they come in
35 contact with the cognate antigen. The interaction of antigen with the antigen receptor on T lymphocytes initiates an ordered series of pleiotropic changes; a process denoted as T lymphocyte activation. T lymphocyte activation is a 7 to 10 day process that results in cell division and the acquisition
40 of immunological functions such as cytotoxicity and the

production of lymphokines that induce antibody production by B lymphocytes and control the growth and differentiation of granulocyte and macrophage precursors. The cytokines produced by activated T lymphocytes act upon other cells of the immune system to coordinate their behavior and bring about an effective immune response.

The initiation of T lymphocyte activation requires a complex interaction of the antigen receptor with the combination of antigen and self-histocompatibility molecules on the surface of antigen-presenting cells. T lymphocytes may also be activated by relatively simple stimuli such as the combination of a calcium ionophore (e.g., ionomycin) and an activator of protein kinase C, such as phorbol myristate acetate (PMA). Several lectins, including phytohemagglutinin (PHA) may also be used to activate T cells (Nowell (1960) Cancer Res. 20: 462).

T lymphocyte activation involves the specific regulation of particular subsets of genes. The transcriptional regulation characteristic of T cell activation begins minutes after the antigen encounter and continues until at least 10 days later. The T lymphocyte activation genes can be grouped according to the time after stimulation at which each gene is transcribed. Early genes are the first subset of T lymphocyte activation genes that is expressed during the activation process. Expression of the early genes triggers the transcriptional modulation of subsequent genes in the activation pathway. Because of the critical role of the T lymphocyte in the immune response, agents that interfere with expression of the early activation genes, such as cyclosporin A and FK506, are effective immunosuppressants.

Transcription of the early genes requires the presence of specific transcription factors, such as NF-AT, which in turn are regulated through interactions with the antigen receptor. These transcription factors are proteins which act through enhancer and promoter elements near the early activation genes to modulate the rate of transcription of these genes. Many of these transcription factors reversibly bind to specific DNA sequences located in and near enhancer elements.

The interleukin-2 (IL-2) gene is a paradigmatic early activation gene. The IL-2 gene product plays a critical role in T lymphocyte proliferation and differentiation. The IL-2 gene is transcriptionally active only in T cells that have been stimulated through the antigen receptor or its associated molecules (Cantrell and Smith (1984) Science 224: 1312). The transcriptional induction of IL-2 in activated T lymphocytes is mediated by a typical early gene transcriptional enhancer that extends from 325 basepairs upstream of the transcriptional start site for the IL-2 gene (Durand et al. (1988) Mol. Cell. Biol. 8: 1715). Other genes known to contain NF-AT recognition sites in their regulatory regions include: γ -interferon, IL-4, GM-CSF, and others. This region, which is referred to herein as the IL-2 enhancer, has been used extensively to dissect the requirements for T lymphocyte activation. An array of transcription factors, including NF-AT, NF κ b, AP-1, Oct-1, and a newly identified protein that associates with Oct-1 called OAP-40, bind to sequences in this region (Ullman et al. (1991) Science 254: 558). These different transcription factors act together to integrate the complex requirements for T lymphocyte activation.

Among the group of transcription factors mentioned above, the presence of NF-AT is characteristic of the transcription events involving early activation genes, in that its recognition sequence is able to enhance transcription of linked heterologous genes in activated T cells of transgenic animals (Verweij et al. (1990) J. Biol. Chem. 265: 15788). The NF-AT sequence element is also the only known transcriptional element in the IL-2 enhancer that has no stimulatory effect on transcription in the absence of physiologic activation of the T lymphocyte through the antigen receptor or through treatment of T cells with the combination of ionomycin and PMA. For example, the NF-AT element enhances transcription of linked sequences in T lymphocytes which have had proper presentation of specific antigen by MHC-matched antigen presenting cells or have been stimulated with the combination of ionomycin/PMA, but not in unstimulated T lymphocytes (Durand et al. (1988) op.cit; Shaw et al. (1988) op.cit; Karttunen and Shastri (1991) Proc.

Natl. Acad. Sci. USA 88: 3972; Verweij et al. (1990) op.cit). Moreover, the NF-AT sequence element naturally enhances transcription of the IL-2 gene only in activated T lymphocytes.

Other elements within the IL-2 enhancer, for example, the NF κ b site or the AP-1 site, activate transcription in response to less specific stimuli, such as tumor necrosis factor α or simply PMA by itself. These compounds do not by themselves activate transcription of the IL-2 gene and other early activation genes, and do not lead to T lymphocyte activation.

Such observations indicate that the expression of certain early genes, such as the interleukin-2 gene may be regulated by the protein complex NF-AT. Data have also indicated that a selective genetic deficiency of NF-AT produces severe combined immunodeficiency (SCID) (Chatilla et al. (1989) New Engl. J. Med. 320: 696).

One of the functional sequences in the IL-2 enhancer is a binding site for a multimeric protein complex, designated NF-AT (nuclear factor of activated T lymphocytes), that functions as a transcriptional regulator of IL-2, IL-4, and other early activation genes (Shaw et al. (1988) Science 241: 202). The NF-AT transcription complex is formed subsequent to a signal from the antigen receptor. Enhancement of transcription of genes adjacent to the NF-AT recognition site requires that the NF-AT complex bind to the recognition site (Shaw et al. (1988) op.cit). Although the molecular makeup of NF-AT is not fully defined, studies have reported that NF-AT can be reconstituted from a ubiquitous nuclear component that requires protein synthesis for induction and a T cell-specific constitutive cytoplasmic component, designated NF-AT_c (Flanagan et al. (1991) Nature 352: 803). This cytoplasmic component, NF-AT_c, associates with the nucleus in response to calcium signalling in a manner that is inhibited by the immunosuppressive drugs cyclosporin A (CsA) and FK506. The nuclear component of NF-AT can be induced with PMA, is not sensitive to CsA or FK506, and can be seen in cells of non-T cell origin such as HeLa and Cos.

Northrop et al. (1993) J. Biol. Chem. 268: 2917 report that the nuclear component of NF-AT contains the phorbol ester-inducible transcription factor, AP-1 (Jun/Fos), and show that antisera to Fos (a component of AP-1) inhibits NF-AT binding to DNA containing a binding site for AP-1. Moreover, Northrop et al. show that NF-AT DNA binding can be reconstituted in vitro using semi-purified AP-1 proteins mixed with cytosol from T lymphocytes, presumably containing NF-AT_c. Northrop et al. also report partial purification of NF-AT_c and report a molecular mass range of approximately 94 to 116 kD as estimated by SDS-polyacrylamide gel electrophoresis.

As noted above, cyclosporin A (CsA) and FK506 are capable of acting as immunosuppressants. These agents inhibit T and B cell activation, mast cell degranulation, and other processes essential to an effective immune response (Borel et al. (1976) Agents Actions 6: 468; Sung et al. (1988) J. Exp. Med. 168: 1539; Gao et al. Nature 336: 176). In T lymphocytes, these drugs disrupt a step in the signal transduction pathway(s) through which the binding of antigen to the T cell antigen receptor produces enhanced transcription of specific cytokine genes involved in the coordination of the immune response. Thus, these agents prevent T lymphocyte activation (Crabtree et al. (1989) Science 243: 355; Schreiber et al. (1989) Science 251:283; Hohman & Hutlsch (1990) New Biol. 2: 663) and act as immunosuppressants.

Putative intracellular receptors for FK506 and CsA have been described and found to be cis-trans prolyl isomerases (Fischer & Bang (1985) Biochim. Biophys. Acta 828: 39; Fischer et al. Nature 337: 476; Handschumacher et al. (1984) Science 226: 544; Lang & Schmid (1988) Nature 331: 453; Standaert et al. (1990) Nature 346: 671). Binding of the drugs inhibits isomerase activity; however, studies with other prolyl isomerase inhibitors (Bierer et al. (1990) Science 250: 556) and analysis of cyclosporin-resistant mutants in yeast suggest that the prevention of T lymphocyte activation results from formation of an inhibitory complex involving the drug and the isomerase (Bierer et al. (1990) Proc. Natl. Acad. Sci. U.S.A. 87: 9231; Tropschug et al. (1989) Nature 342: 953), and not

from inhibition of the isomerase activity per se. CsA and FK-506 prevent T cell proliferation by inhibiting a calcium-dependent signalling event required for the induction of interleukin-2 transcription.

5 Calcineurin, a calmodulin-dependent protein phosphatase which occurs in various isoforms, has been identified as a critical component of T cell activation through the signal transduction pathway leading to transcriptional activation of NF-AT-dependent genes, such as lymphokine genes
10 (Liu et al. (1991) Cell 66: 807; Clipstone and Crabtree (1992) Nature 357: 695; O'Keefe et al. (1992) Nature 357: 692)

Transcriptional enhancement involving NF-AT recognition sequences is completely blocked in T cells treated with efficacious concentrations of cyclosporin A or FK506, with
15 little or no specific effect on transcriptional enhancement involving recognition sites for other transcription factors, such as AP-1 and NF- κ B (Shaw et al. (1988) op.cit.; Emmel et al. (1989) Science 246: 1617; Mattila et al. (1990) EMBO J. 9: 4425). This blockage can be overcome, at least partially, by
20 the expression of hyperphysiological amounts of calcineurin (Clipstone and Crabtree (1992) op.cit.).

Unfortunately, while both cyclosporin A and FK506 are potent immunosuppressive agents, both drugs possess detrimental properties. For example, cyclosporin elicits adverse reactions
25 including renal dysfunction, tremors, nausea and hypertension. Indeed, for many years researchers have attempted to develop superior replacements, with FK506 being the most recent candidate. However, without understanding the mechanisms by which cyclosporin (or FK506) functions at the intracellular
30 level, developing improved immunosuppressants represents an extremely difficult research effort with a limited likelihood of success.

Thus, there exists a significant need to understand the functional basis of T cell activation involving NF-AT,
35 particularly with regard to the mechanism by which these immunosuppressants such as CsA and FK506 inhibit transcription of the early activation genes. With such knowledge, improved assays for screening drug candidates would be feasible, which

could in turn dramatically enhance the search process. Modulation of the immune system, especially modulation of T cell activation, also may be effected by directly altering the amount or activity of NF-AT. The present invention fulfills these and other needs.

The references discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention.

SUMMARY OF THE INVENTION

The present invention provides several novel methods and compositions for modulating the immune response and for screening for modulators of the immune response. These methods utilize polynucleotide sequences encoding NF-AT_c recombinant proteins and complementary polynucleotides which are substantially identical to NF-AT_c polynucleotide sequences.

In one aspect of the invention, NF-AT_c polypeptides and compositions thereof are provided. NF-AT_c polypeptides comprise polypeptide sequences which are substantially identical to a sequence shown in Fig. 1 or a cognate NF-AT_c gene sequence.

Nucleic acid sequences encoding NF-AT_c are provided. The characteristics of the cloned sequences are given, including the nucleotide and predicted amino acid sequence in Fig. 1. Polynucleotides comprising these sequences can serve as templates for the recombinant expression of quantities of NF-AT_c polypeptides, such as human NF-AT_c and murine NF-AT_c. Polynucleotides comprising these sequences can also serve as probes for nucleic acid hybridization to detect the transcription and mRNA abundance of NF-AT_c mRNA in individual lymphocytes (or other cell types) by in situ hybridization, and in specific lymphocyte populations by Northern blot analysis and/or by in situ hybridization (Alwine et al. (1977) Proc. Natl. Acad. Sci. U.S.A. 74: 5350) and/or PCR amplification and/or LCR detection. Such recombinant polypeptides and nucleic acid hybridization probes have utility for in vitro

screening methods for immunomodulatory agents and for diagnosis and treatment of pathological conditions and genetic diseases, such as transplant rejection reactions, T cell-mediated immune responses, lymphocytic leukemias (e.g., T cell leukemia or lymphoma) wherein NF-AT activity contributes to disease processes, autoimmune disease, arthritis, and the like.

In one embodiment, candidate immunomodulatory agents are identified by their ability to block the binding of a NF-AT_c polypeptide to other components of NF-AT (e.g., AP-1) and/or to block the binding of NF-AT to DNA having an NF-AT recognition site. The DNA preferably includes one or more NF-AT binding sites at which a NF-AT protein complex specifically binds. One means for detecting binding of a NF-AT protein comprising NF-AT_c to DNA is to immobilize the DNA, such as by covalent or noncovalent chemical linkage to a solid support, and to contact the immobilized DNA with a NF-AT protein complex comprising a NF-AT_c polypeptide that has been labeled with a detectable marker (e.g., by incorporation of radiolabeled amino acid). Such contacting is typically performed in aqueous conditions which permit binding of a NF-AT protein to a target DNA containing a NF-AT binding sequence. Binding of the labeled NF-AT to the immobilized DNA is measured by determining the extent to which the labeled NF-AT_c polypeptide is immobilized as a result of a specific binding interaction. Such specific binding may be reversible, or may be optionally irreversible if a cross-linking agent is added in appropriate experimental conditions.

In one aspect, candidate immunomodulatory agents are identified as being agents capable of inhibiting (or enhancing) intermolecular binding between NF-AT_c and other polypeptides which comprise a NF-AT complex (e.g., AP-1, JunB, etc.). The invention provides methods and compositions for screening libraries of agents for the capacity to interfere with binding of NF-AT_c to other NF-AT polypeptide species under aqueous binding conditions. Typically, at least either NF-AT_c and/or another NF-AT polypeptide species is labeled with a detectable label and intermolecular binding between NF-AT_c and other NF-

AT polypeptide species is detected by the amount of labeled species captured in NF-AT complexes and the like.

The invention also provides antisense polynucleotides complementary to NF-AT_c sequences which are employed to inhibit transcription and/or translation of the cognate mRNA species and thereby effect a reduction in the amount of the respective NF-AT_c protein in a cell (e.g., a T lymphocyte of a patient). Such antisense polynucleotides can function as immunomodulatory drugs by inhibiting the formation of NF-AT protein required for T cell activation.

In a variation of the invention, polynucleotides of the invention are employed for diagnosis of pathological conditions or genetic disease that involve T cell neoplasms or T cell hyperfunction or hypofunction, and more specifically conditions and diseases that involve alterations in the structure or abundance of NF-AT_c polypeptide, NF-AT_c polynucleotide sequence, or structure of the NF-AT_c gene or flanking region(s).

The invention also provides antibodies which bind to NF-AT_c with an affinity of about at least $1 \times 10^7 \text{ M}^{-1}$ and which lack specific high affinity binding for other proteins present in activated T cells. Such antibodies can be used as diagnostic reagents to identify T cells (e.g., activatable T cells) in a cellular sample from a patient (e.g., a lymphocyte sample, a solid tissue biopsy) as being cells which contain an increased amount of NF-AT_c protein determined by standardization of the assay to be diagnostic for activated T cells. Frequently, anti-NF-AT_c antibodies are included as diagnostic reagents for immunohistopathology staining of cellular samples in situ. Additionally, anti-NF-AT_c antibodies may be used therapeutically by targeted delivery to T cells (e.g., by cationization or by liposome/immunoliposome delivery).

The invention also provides NF-AT_c polynucleotide probes for diagnosis of neoplasia or immune status by detection of NF-AT_c mRNA in cells explanted from a patient, or detection of a pathognomonic NF-AT_c allele (e.g., by RFLP or allele-specific PCR analysis). A pathognomonic NF-AT_c allele is an

allele which is statistically correlated with the presence of a predetermined disease or propensity to develop a disease. Typically, the detection will be by in situ hybridization using a labeled (e.g., ^{32}P , ^{35}S , ^{14}C , ^3H , fluorescent, biotinylated, digoxigeninylated) NF-AT_c polynucleotide, although Northern blotting, dot blotting, or solution hybridization on bulk RNA or poly A⁺ RNA isolated from a cell sample may be used, as may PCR amplification using NF-AT_c-specific primers. Cells which contain an increased amount of NF-AT_c mRNA as compared to standard control values for cells or cell types other than activated T cells or activatable T cells will be thereby identified as activated T cells or activatable T cells. Similarly, the detection of pathognomonic rearrangements or amplification of the NF-AT_c locus or closely linked loci in a cell sample will identify the presence of a pathological condition or a predisposition to developing a pathological condition (e.g., cancer, genetic disease).

The present invention also provides a method for diagnosing T cell hypofunction or hyperfunction in a human patient, wherein a diagnostic assay (e.g., immunohistochemical staining of fixed lymphocytic cells by an antibody that specifically binds human NF-AT_c) is used to determine if a predetermined pathognomonic concentration of NF-AT_c protein or NF-AT_c mRNA is present in a biological sample from a human patient; if the assay indicates the presence of NF-AT_c protein or NF-AT_c mRNA at or above such predetermined pathognomonic concentration, the patient is diagnosed as having T cell hyperfunction or hypofunction condition, or transplant rejection and the like.

All publications and patent applications herein are incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 (panels a and b) shows the nucleotide sequence of the human NF-AT_c cDNA and the deduced amino acid sequence. Nucleotide sequence and complete predicted amino acid sequence

of NF-AT_c from human T lymphocytes. Bovine peptides (sequences not completely conserved) identified in the predicted human sequence are underlined. The cDNA ends in a canonical polyadenylation signal and a poly A tail.

5 Fig. 2 shows the expression of NF-AT_c protein in T cells (Jurkat) and non-T cells (Cos).

Fig. 3 (panels a and b) show that the NF-AT_c cDNA clone encodes a protein that activates transcription from an NF-AT site and is capable of activating the IL-2 promoter in
10 non-T cells.

Fig. 4 shows homology between NF-AT_c, NF-AT_p, and Rel family members. The protein sequences of murine NF-AT_p and the Rel proteins Dorsal (the Drosophila axis-determining protein), human c-Rel, NF- κ B p50, and NF- κ B p65 are aligned to the
15 sequence of NF-AT_c. Numbering is with respect to NF-AT_c. Identity to NF-AT_c, open boxes; similarity in known residue function or structure, shaded areas. Stars indicate regions in which NF-AT_c has: 1) a charge reversal relative to the majority of other Rel proteins, or has 2) replaced a potential salt
20 bridge residue with a histidine or other chelating residue. Lower portion shows a schematic of NF-AT_c and NF-AT_p.

Fig. 5 (panels a-c). Panel a: Ribonuclease protection for human NF-AT_c with RNA from Jurkat cells (lanes 1-6) or Hela cells (lane 7). The expected specific
25 ribonuclease-resistant fragment is 304 nucleotides (arrow). Hela cells were non-stimulated. Jurkat cells were either non-stimulated or stimulated with 20ng/ml PMA and 2uM ionomycin for 3 hours, plus or minus 100ng/ml CsA added at the indicated times after stimulation. Panel b: RNA from the following human
30 cells: KJ (preB cell ALL), JD-1 (B cell lineage ALL), K562 (erythroleukemia cell line), CML (bone marrow cells from a patient with a myeloid leukemia), human muscle tissue, Hep G2 (liver cell line), HPB ALL (T cell line, nonstimulated or stimulated with 2ug/mi PHA and 50ng/ml PMA for 30 minutes), and
35 Hela cells analyzed by ribonuclease protection. A longer exposure of this gel indicates that the K562 cell line contains a small amount of NF-AT_c transcript. Panel c: NF-AT_c (upper panel) and NF-AT_p (lower panel) mRNA expression in mouse

tissues and a skin tumor derived from NF-AT-Tag transgenic mice (Verweij et al. (1990) *J. Biol. Chem* 265: 15788-15795). Cells were either non-stimulated or stimulated with 20ng/ml PMA and 2uM ionomycin for 3 hours. RNA was measured by quantitative ribonuclease protection using murine cDNA probes. The predicted size of the fragment homologous to the probe is indicated by the arrows.

Fig. 6 (panels a-d). Panel a: Cos cells and Jurkat cells were transfected with reporter constructs for NF-AT or HNF-1 (β 28). Co-transfected expression vectors for NF-AT_c (+NF-AT) or HNF-1 α (+HNF-1) were included where indicated, otherwise empty pBJ5 vector was included. Cells were stimulated as indicated: PMA, P + I (PMA plus ionomycin). Panel b: Cos cells were transfected with IL-2 luciferase and with expression vectors as in a. Stimulations were as in a. Data in a and b are expressed as fold induction of luciferase activity over nonstimulated value with empty pBJ5 vector. Bars represent mean and range of 2-3 independent transfections. Panel c: Expression of NF-AT_c in Cos cells gives rise to specific DNA binding activity. Gel mobility shifts using nuclear extracts from Cos cells transfected with pBJ5 (lanes 1 and 3), with NF-AT_c (lanes 2 and 4-7), from non-transfected Jurkat cells (lanes 8-11) or using cytosols from pBJ5- or NF-AT_c-transfected Cos cells (lanes 12-13, 15-16) combined with Hela nuclear extract (lanes 15-16). Lane 14, Hela nuclear extract alone. Labeled AP-1 (lanes 1-2) or NF-AT (lanes 3-16) probes and cold competitor oligonucleotides are indicated. Arrows indicate specific AP-1 and NF-AT complexes. Panel d: Antisera induced supershift of NF-AT. NF-AT and AP-1 gel mobility shifts using nuclear extracts from stimulated Jurkat cells or murine thymocytes. Either no antisera, preimmune, or one of two different immune antisera was included as indicated. Arrows indicate specific NF-AT or AP1 complexes or supershifted NF-AT complexes (*).

Fig. 7 shows dominant-negative NF-AT_c. Jurkat Tag cells were transfected with vector plasmid (control) or with the dominant negative NF-AT_c plasmid, plus the indicated secreted alkaline phosphatase reporter plasmid. Transfected

cells were transferred to fresh culture medium 24 hours after transfection and secreted alkaline phosphatase activity was measured (Clipstone and Crabtree (1992) Nature 357: 695-698) 16 to 24 hours later, after stimulation with 1 μ M ionomycin plus 5 20 ng/ml PMA (NF-AT and IL-2 reporters), 20 ng/ml PMA alone (API reporter) or no stimulation (RSV reporter). Bars indicate, secreted alkaline phosphatase activity from cells transfected with the dominant negative NF-AT_c as a percentage of the activity from cells transfected in parallel with control 10 plasmid, and represent data obtained from (n) independent transfections. The dominant negative NF-AT_c consists of a carboxy terminal truncation of the epitope tagged NF-AT_c expression plasmid extending to the PvuII site at amino acid 463.

15 Fig. 8 shows changes in mobility of epitope tagged NF-AT_c expressed in Jurkat cells. Cells were transfected with NF-AT_c as in Fig. 2 and stimulated as shown for 2 hrs plus or minus 100ng/ml CSA. Whole cell lysates were analyzed by western blotting as in Fig. 2.

20

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar 25 or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described. For purposes of the present invention, the following terms are defined below.

30 As used herein, the twenty conventional amino acids and their abbreviations follow conventional usage (Immunology - A Synthesis, 2nd Edition, E.S. Golub and D.R. Gren, Eds., Sinauer Associates, Sunderland, Massachusetts (1991), which is incorporated herein by reference). Stereoisomers (e.g., D-amino acids) of the twenty conventional amino acids, unnatural amino acids such as α,α -disubstituted amino acids, N-alkyl amino acids, lactic acid, and other unconventional amino acids 35 may also be suitable components for polypeptides of the present

invention. Examples of unconventional amino acids include: 4-hydroxyproline, γ -carboxyglutamate, ϵ -N,N,N-trimethyllysine, ϵ -N-acetyllysine, O-phosphoserine, N-acetylserine, N-formylmethionine, 3-methylhistidine, 5-hydroxylysine, ω -N-methylarginine, and other similar amino acids and imino acids (e.g., 4-hydroxyproline). In the polypeptide notation used herein, the lefthand direction is the amino terminal direction and the righthand direction is the carboxy-terminal direction, in accordance with standard usage and convention. Similarly, unless specified otherwise, the lefthand end of single-stranded polynucleotide sequences is the 5' end; the lefthand direction of double-stranded polynucleotide sequences is referred to as the 5' direction. The direction of 5' to 3' addition of nascent RNA transcripts is referred to as the transcription direction; sequence regions on the DNA strand having the same sequence as the RNA and which are 5' to the 5' end of the RNA transcript are referred to as "upstream sequences"; sequence regions on the DNA strand having the same sequence as the RNA and which are 3' to the 3' end of the RNA transcript are referred to as "downstream sequences".

The term "naturally-occurring" as used herein as applied to an object refers to the fact that an object can be found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses) that can be isolated from a source in nature and which has not been intentionally modified by man in the laboratory is naturally-occurring.

The term "corresponds to" is used herein to mean that a polynucleotide sequence is homologous (i.e., is identical, not strictly evolutionarily related) to all or a portion of a reference polynucleotide sequence, or that a polypeptide sequence is identical to a reference polypeptide sequence. In contradistinction, the term "complementary to" is used herein to mean that the complementary sequence is homologous to all or a portion of a reference polynucleotide sequence. For illustration, the nucleotide sequence "TATAC" corresponds to a reference sequence "TATAC" and is complementary to a reference sequence "GTATA".

The following terms are used to describe the sequence relationships between two or more polynucleotides: "reference sequence", "comparison window", "sequence identity", "percentage of sequence identity", and "substantial identity".

- 5 A "reference sequence" is a defined sequence used as a basis for a sequence comparison; a reference sequence may be a subset of a larger sequence, for example, as a segment of a full-length cDNA or gene sequence given in a sequence listing, such as a polynucleotide sequence of Fig. 1, or may comprise a
- 10 complete cDNA or gene sequence. Generally, a reference sequence is at least 20 nucleotides in length, frequently at least 25 nucleotides in length, and often at least 50 nucleotides in length. Since two polynucleotides may each (1) comprise a sequence (i.e., a portion of the complete
- 15 polynucleotide sequence) that is similar between the two polynucleotides, and (2) may further comprise a sequence that is divergent between the two polynucleotides, sequence comparisons between two (or more) polynucleotides are typically performed by comparing sequences of the two polynucleotides
- 20 over a "comparison window" to identify and compare local regions of sequence similarity. A "comparison window", as used herein, refers to a conceptual segment of at least 20 contiguous nucleotide positions wherein a polynucleotide sequence may be compared to a reference sequence of at least 20
- 25 contiguous nucleotides and wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) of 20 percent or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two
- 30 sequences. Optimal alignment of sequences for aligning a comparison window may be conducted by the local homology algorithm of Smith and Waterman (1981) Adv. Appl. Math. 2: 482, by the homology alignment algorithm of Needleman and Wunsch (1970) J. Mol. Biol. 48: 443, by the search for similarity
- 35 method of Pearson and Lipman (1988) Proc. Natl. Acad. Sci. (U.S.A.) 85: 2444, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group,

575 Science Dr., Madison, WI), or by inspection, and the best alignment (i.e., resulting in the highest percentage of homology over the comparison window) generated by the various methods is selected. The term "sequence identity" means that two polynucleotide sequences are identical (i.e., on a nucleotide-by-nucleotide basis) over the window of comparison. The term "percentage of sequence identity" is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (e.g., A, T, C, G, U, or I) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity. The terms "substantial identity" as used herein denotes a characteristic of a polynucleotide sequence, wherein the polynucleotide comprises a sequence that has at least 85 percent sequence identity, preferably at least 90 to 95 percent sequence identity, more usually at least 99 percent sequence identity as compared to a reference sequence over a comparison window of at least 20 nucleotide positions, frequently over a window of at least 25-50 nucleotides, wherein the percentage of sequence identity is calculated by comparing the reference sequence to the polynucleotide sequence which may include deletions or additions which total 20 percent or less of the reference sequence over the window of comparison. The reference sequence may be a subset of a larger sequence, for example, as a segment of the full-length human NF-AT_c polynucleotide sequence shown in Fig. 1 or the full-length murine or bovine NF-AT_c cDNA sequence.

As applied to polypeptides, the term "substantial identity" means that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 80 percent sequence identity, preferably at least 90 percent sequence identity, more preferably at least 95 percent sequence identity or more (e.g., 99 percent sequence identity). Preferably, residue positions which are not identical differ by conservative amino acid

substitutions. Conservative amino acid substitutions refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side chains is cysteine and methionine. Preferred conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, and asparagine-glutamine.

The term "NF-AT_c native protein" and "full-length NF-AT_c protein" as used herein refers to a naturally-occurring NF-AT_c polypeptide corresponding to the deduced amino acid sequence shown in Fig. 1 or corresponding to the deduced amino acid sequence of a cognate full-length cDNA. Also for example, a native NF-AT_c protein present in naturally-occurring lymphocytes which express the NF-AT_c gene are considered full-length NF-AT_c proteins.

The term "NF-AT_c fragment" as used herein refers to a polypeptide that has an amino-terminal and/or carboxy-terminal deletion, but where the remaining amino acid sequence is identical to the corresponding positions in the NF-AT_c sequence deduced from a full-length cDNA sequence (e.g., the cDNA sequence shown in Fig. 1). NF-AT_c fragments typically are at least 14 amino acids long, preferably at least 20 amino acids long, usually at least 50 amino acids long or longer.

The term "NF-AT_c analog" as used herein refers to polypeptides which are comprised of a segment of at least 25 amino acids that has substantial identity to a portion of the deduced amino acid sequence shown in Fig. 1, and which has at least one of the following properties: (1) binding to other NF-AT proteins (e.g., AP-1) under suitable binding conditions, or (2) ability to localize to the nucleus upon T cell activation.

Typically, NF-AT_c analog polypeptides comprise a conservative amino acid substitution (or addition or deletion) with respect to the naturally-occurring sequence. NF-AT_c analogs typically are at least 20 amino acids long, preferably at least 50 amino acids long or longer, most usually being as long as full-length naturally-occurring NF-AT_c (e.g., as shown in Fig. 1). Some NF-AT_c analogs may lack biological activity but may still be employed for various uses, such as for raising antibodies to NF-AT_c epitopes, as an immunological reagent to detect and/or purify α-NF-AT_c antibodies by affinity chromatography, or as a competitive or noncompetitive agonist, antagonist, or partial agonist of native NF-AT_c protein function.

The term "NF-AT_c polypeptide" is used herein as a generic term to refer to native protein, fragments, or analogs of NF-AT_c. Hence, native NF-AT_c, fragments of NF-AT_c, and analogs of NF-AT_c are species of the NF-AT_c polypeptide genus. Preferred NF-AT_c polypeptides include: the human full-length NF-AT_c protein comprising the polypeptide sequence shown in Fig. 1, or polypeptides consisting essentially of a sequence shown in Table II.

The term "cognate" as used herein refers to a gene sequence that is evolutionarily and functionally related between species. For example but not limitation, in the human genome, the human CD4 gene is the cognate gene to the mouse CD4 gene, since the sequences and structures of these two genes indicate that they are highly homologous and both genes encode a protein which functions in signaling T cell activation through MHC class II-restricted antigen recognition. Thus, the cognate murine gene to the human NF-AT_c gene is the murine gene which encodes an expressed protein which has the greatest degree of sequence identity to the human NF-AT_c protein and which exhibits an expression pattern similar to that of the human NF-AT_c (e.g., expressed in T lineage cells). Preferred cognate NF-AT_c genes are: rat NF-AT_c, rabbit NF-AT_c, canine NF-AT_c, nonhuman primate NF-AT_c, porcine NF-AT_c, bovine NF-AT_c, and hamster NF-AT_c.

The term "NF-AT_c-dependent gene" is used herein to refer to genes which: (1) have a NF-AT binding site (a site

which can be specifically footprinted by NF-AT under suitable binding conditions) within about 10 kilobases of the first coding sequence of said gene, and (2) manifest an altered rate of transcription, either increased or decreased, from a major or minor transcriptional start site for said gene, wherein such alteration in transcriptional rate correlates with the presence of NF-AT_c polypeptide in NF-AT complexes, such as in an activated T cell.

The term "altered ability to modulate" is used herein to refer to the capacity to either enhance transcription or inhibit transcription of a gene; such enhancement or inhibition may be contingent on the occurrence of a specific event, such as T cell stimulation. This alteration will be manifest as an inhibition of the transcriptional enhancement of the IL-2 gene that normally ensues following T cell stimulation. The altered ability to modulate transcriptional enhancement or inhibition may affect the inducible transcription of a gene, such as in the just-cited IL-2 example, or may effect the basal level transcription of a gene, or both.

The term "agent" is used herein to denote a chemical compound, a mixture of chemical compounds, a biological macromolecule, or an extract made from biological materials such as bacteria, plants, fungi, or animal (particularly mammalian) cells or tissues. Agents are evaluated for potential activity as immunomodulatory agents (e.g., immunosuppressants) by inclusion in screening assays described hereinbelow.

The term "candidate immunomodulatory agent" is used herein to refer to an agent which is identified by one or more screening method(s) of the invention as a putative immunomodulatory agent. Some candidate immunomodulatory agents may have therapeutic potential as drugs for human use.

As used herein, the terms "label" or "labeled" refers to incorporation of a detectable marker, e.g., by incorporation of a radiolabeled amino acid or attachment to a polypeptide of biotinyl moieties that can be detected by marked avidin (e.g., streptavidin containing a fluorescent marker or enzymatic activity that can be detected by optical or

colorimetric methods). Various methods of labeling polypeptides and glycoproteins are known in the art and may be used. Examples of labels for polypeptides include, but are not limited to, the following: radioisotopes (e.g., ^3H , ^{14}C , ^{35}S , ^{125}I , ^{131}I), fluorescent labels (e.g., FITC, rhodamine, lanthanide phosphors), enzymatic labels (e.g., horseradish peroxidase, β -galactosidase, luciferase, alkaline phosphatase), biotinyl groups, predetermined polypeptide epitopes recognized by a secondary reporter (e.g., leucine zipper pair sequences, binding sites for secondary antibodies, metal binding domains, epitope tags). In some embodiments, labels are attached by spacer arms of various lengths to reduce potential steric hindrance.

As used herein, "substantially pure" means an object species is the predominant species present (i.e., on a molar basis it is more abundant than any other individual species in the composition), and preferably a substantially purified fraction is a composition wherein the object species comprises at least about 50 percent (on a molar basis) of all macromolecular species present. Generally, a substantially pure composition will comprise more than about 80 to 90 percent of all macromolecular species present in the composition. Most preferably, the object species is purified to essential homogeneity (contaminant species cannot be detected in the composition by conventional detection methods) wherein the composition consists essentially of a single macromolecular species. A nucleic acid is "isolated" or "rendered substantially pure" when purified away from other naturally occurring cellular components or other contaminants, e.g., other cellular nucleic acids or proteins, by standard techniques, including alkaline/SDS treatment, CsCl banding, column chromatography, agarose gel electrophoresis and others well known in the art, or by replication in a heterologous host cell (e.g., a human NF-AT_c polynucleotide in a non-human cell). A polypeptide is "isolated" when purified away from other cellular components or contaminants which naturally occur in conjunction with the polypeptide species; for example, a human NF-AT_c polypeptide which is substantially purified of other

human proteins is isolated, as is a human NF-AT_c polypeptide expressed in a non-human cell (e.g., COS cell, murine cell, CHO cell, yeast, or bacterium).

As used herein the terms "pathognomonic concentration", "pathognomonic amount", and "pathognomonic staining pattern" refer to a concentration, amount, or localization pattern, respectively, of a NF-AT_c protein or mRNA in a sample, that indicates the presence of a hypofunctional or hyperfunctional T cell condition or a predisposition to developing a disease, such as graft rejection. A pathognomonic amount is an amount of a NF-AT_c protein or NF-AT_c mRNA in a cell or cellular sample that falls outside the range of normal clinical values that is established by prospective and/or retrospective statistical clinical studies. Generally, an individual having a neoplastic disease (e.g., lymphocytic leukemia) or T cell-mediated immune response will exhibit an amount of NF-AT_c protein or mRNA in a cell or tissue sample that is higher than the range of concentrations that characterize normal, undiseased individuals; typically the pathognomonic concentration is at least about one standard deviation above the mean normal value, more usually it is at least about two standard deviations or more above the mean normal value. However, essentially all clinical diagnostic tests produce some percentage of false positives and false negatives. The sensitivity and selectivity of the diagnostic assay must be sufficient to satisfy the diagnostic objective and any relevant regulatory requirements. In general, the diagnostic methods of the invention are used to identify individuals as disease candidates, providing an additional parameter in a differential diagnosis of disease made by a competent health professional.

DETAILED DESCRIPTION

Generally, the nomenclature used hereafter and the laboratory procedures in cell culture, molecular genetics, and nucleic acid chemistry and hybridization described below are those well known and commonly employed in the art. Standard techniques are used for recombinant nucleic acid methods,

polynucleotide synthesis, and microbial culture and transformation (e.g., electroporation, lipofection). Generally enzymatic reactions and purification steps are performed according to the manufacturer's specifications. The techniques and procedures are generally performed according to conventional methods in the art and various general references (see, generally, Sambrook et al. Molecular Cloning: A Laboratory Manual, 2d ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., which is incorporated herein by reference) which are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

Oligonucleotides can be synthesized on an Applied Bio Systems oligonucleotide synthesizer according to specifications provided by the manufacturer.

Methods for PCR amplification are described in the art (PCR Technology: Principles and Applications for DNA Amplification ed. HA Erlich, Freeman Press, New York, NY (1992); PCR Protocols: A Guide to Methods and Applications, eds. Innis, Gelfland, Snisky, and White, Academic Press, San Diego, CA (1990); Mattila et al. (1991) Nucleic Acids Res. **19**: 4967; Eckert, K.A. and Kunkel, T.A. (1991) PCR Methods and Applications **1**: 17; PCR, eds. McPherson, Quirk, and Taylor, IRL Press, Oxford; and U.S. Patent 4,683,202, which are incorporated herein by reference).

Commonly assigned application U.S.S.N. 07/749,385 filed 22 August 1991 is incorporated herein by reference.

Cloning of NF-AT_c Polynucleotides

Genomic or cDNA clones encoding NF-AT_c may be isolated from clone libraries (e.g., available from Clontech, Palo Alto, CA) using hybridization probes designed on the basis of the nucleotide sequences shown in Fig. 1 and using conventional hybridization screening methods (e.g., Benton WD and Davis RW (1977) Science **196**: 180; Goodspeed et al. (1989) Gene **76**: 1; Dunn et al. (1989) J. Biol. Chem. **264**: 13057).

Where a cDNA clone is desired, clone libraries containing cDNA derived from T cell mRNA is preferred. Typically, a suitable hybridization probe will comprise at least 35-50 contiguous nucleotides of the nucleotide sequence shown in Fig. 1, usually representing a coding portion of the NF-AT_c mRNA sequence (i.e., from about nucleotide 240 to about nucleotide 2390 according to the numbering convention shown in Fig. 1). Alternatively, synthetic polynucleotide sequences corresponding to all or part of the sequences shown in Fig. 1 may be constructed by chemical synthesis of oligonucleotides. Additionally, polymerase chain reaction (PCR) using primers based on the sequence data disclosed in Fig. 1 may be used to amplify DNA fragments from genomic DNA, mRNA pools, or from cDNA clone libraries. U.S. Patents 4,683,195 and 4,683,202 describe the PCR method. Additionally, PCR methods employing one primer that is based on the sequence data disclosed in Fig. 1 and a second primer that is not based on that sequence data may be used. For example, a second primer that is homologous to or complementary to a polyadenylation segment may be used. In an embodiment, a polynucleotide comprising the 2742 nucleotide-long sequence of Fig. 1 can be used. Alternative polynucleotides encoding the 716 amino acid sequence of Fig. 1 can also be readily constructed by those of skill in the art by using the degeneracy of the genetic code. Polynucleotides encoding amino acids 418 to 710 of the NF-AT_c sequence of Fig. 1 can also be constructed by those of skill in the art.

It is apparent to one of skill in the art that nucleotide substitutions, deletions, and additions may be incorporated into the polynucleotides of the invention. Nucleotide sequence variation may result from sequence polymorphisms of various NF-AT_c alleles, minor sequencing errors, and the like. However, such nucleotide substitutions, deletions, and additions should not substantially disrupt the ability of the polynucleotide to hybridize to one of the polynucleotide sequences shown in Fig. 1 under hybridization conditions that are sufficiently stringent to result in specific hybridization.

Specific hybridization is defined herein as the formation of hybrids between a probe polynucleotide (e.g., a polynucleotide of the invention which may include substitutions, deletion, and/or additions) and a specific target polynucleotide (e.g., a polynucleotide having the sequence in Fig. 1), wherein the probe preferentially hybridizes to the specific target such that, for example, a single band corresponding to NF-AT_c mRNA (or bands corresponding to multiple alternative splicing products of the NF-AT_c gene) can be identified on a Northern blot of RNA prepared from a suitable cell source (e.g., a T cell expressing NF-AT_c). Polynucleotides of the invention and recombinantly produced NF-AT_c, and fragments or analogs thereof, may be prepared on the basis of the sequence data provided in Fig. 1 according to methods known in the art and described in Maniatis et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., (1989), Cold Spring Harbor, N.Y. and Berger and Kimmel, Methods in Enzymology, Volume 152, Guide to Molecular Cloning Techniques (1987), Academic Press, Inc., San Diego, CA, which are incorporated herein by reference.

NF-AT_c polynucleotides may be short oligonucleotides (e.g., 25-100 bases long), such as for use as hybridization probes and PCR (or LCR) primers. NF-AT_c polynucleotide sequences may also comprise part of a larger polynucleotide (e.g., a cloning vector comprising a NF-AT_c clone) and may be fused, by polynucleotide linkage, in frame with another polynucleotide sequence encoding a different protein (e.g., glutathione S-transferase or β -galactosidase) for encoding expression of a fusion protein. Typically, NF-AT_c polynucleotides comprise at least 25 consecutive nucleotides which are substantially identical to a naturally-occurring NF-AT_c sequence (e.g., Fig. 1), more usually NF-AT_c polynucleotides comprise at least 50 to 100 consecutive nucleotides which are substantially identical to a naturally-occurring NF-AT_c sequence. However, it will be recognized by those of skill that the minimum length of a NF-AT_c polynucleotide required for specific hybridization to a NF-AT_c target sequence will depend on several factors: G/C content,

positioning of mismatched bases (if any), degree of uniqueness of the sequence as compared to the population of target polynucleotides, and chemical nature of the polynucleotide (e.g., methylphosphonate backbone, phosphorothiolate, etc.), among others.

For example but not limitation, suitable hybridization probes for detecting and/or quantifying the presence of NF-AT_c mRNA in a sample generally comprise at least one, preferably at least two, often three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, fifteen, sixteen, seventeen, eighteen or nineteen, and more preferably all 20 of the following human NF-AT_c sequences shown in Table I, or their complements:

Table I: Selected Human NF-AT_c Polynucleotide Sequences

5'-TTC CTC CGG GGC GCG CGG CGT GAG CCC GGG GCG AGG-3';
 5'-CAG CGC GGG GCG GCC ACT TCT CCT GTG CCT CCG CCC GCT GCT-3';
 5'-GCC GCG CGG ATG CCA AGC ACC AGC TTT CCA GTC CCT TCC AAG-3';
 5'-CCA ACG TCA GCC CCG CCC TGC CGC TCC CCA CGG CGC ACT CCA-3';
 5'-TTC AGA CCT CCA CAC CGG GCA TCA TCC CGC CGG CGG-3';
 5'-GCC ACA CCA GGC CTG ATG GGG CCC CTG CCC TGG AGA GTC CTC-3';
 5'-AGT CTG CCC AGC CTG GAG GCC TAC AGA GAC CCC TCG TGC CTG-3';
 5'-GTG TCT CCC AAG ACC ACG GAC CCC GAG GAG GGC TTT CCC-3';
 5'-AGC TGG CTG GGT GCC CGC TCC TCC AGA CCC GCG TCC CCT TGC-3';
 5'-TAC AGC CTC AAC GGC CGG CAG CCG CCC TAC TCA CCC CAC CAC-3';
 5'-GAC CAC CGA CAG CAG CCT GGA CCT GGG AGA TGG CGT CCC TGT-3';
 5'-CCT GGG CAG CCC CCC GCC CCC GGC CGA CTT CGC GCC CGA AGA-3';
 5'-GCT CCC CTA CCA GTG GCG AAG CCC AAG CCC CTG TCC CCT ACG-3';
 5'-CTT CGG ATT GAG GTG CAG CCC AAG TCC CAC CAC CGA GCC CAC-3';
 5'-CAT GGC TAC TTG GAG AAT GAG CCG CTG ATG CTG CAG CTT TTC-3';
 5'-AAG ACC GTG TCC ACC ACC AGC CAC GAG GCT ATC CTC TCC AAC-3';
 5'-TCA GCT CAG GAG CTG CCT CTG GTG GAG AAG CAG AGC ACG GAC-3';
 5'-AAC GCC ATC TTT CTA ACC GTA AGC CGT GAA CAT GAG CGC G-3';
 5'-AGA AAC GAC GTC GCC GTA AAG CAG CGT GGC GTG TGG CA-3'; and
 5'-GCA TAC TCA GAT AGT CAC GGT TAT TTT GCT TCT TGC GAA TG-3'.

Such polypeptides are typically less than 100 kb, usually less than 10,000 nucleotides, frequently less than 5,000 nucleotides, and generally less than 3000 nucleotides, such as

approximately 2750 nucleotides or approximately 2140 nucleotides, and the like.

Also for example but not limitation, the following pair of PCR primers (amplimers) may be used to amplify murine or human NF-AT_c sequences (e.g., by reverse transcriptase initiated PCR of RNA from NF-AT_c expressing cells):

(forward) 5'-AGGGCGCGGGCACCAGGGGCGGGGCAGGGCTCGGAG-3'

(reverse) 5'-GCAAGAAGCAAAATAACCGTGACTATCTGAGTATGC-3'

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If desired, PCR amplimers for amplifying substantially full-length cDNA copies may be selected at the discretion of the practitioner. Similarly, amplimers to amplify single NF-AT_c exons or portions of the NF-AT_c gene (murine or human) may be selected.

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Each of these sequences may be used as hybridization probes or PCR amplimers to detect the presence of NF-AT_c mRNA, for example to diagnose a disease characterized by the presence of an elevated NF-AT_c mRNA level in lymphocytes, or to perform tissue typing (i.e., identify tissues characterized by the expression of NF-AT_c mRNA), and the like. The sequences may also be used for detecting genomic NF-AT_c gene sequences in a DNA sample, such as for forensic DNA analysis (e.g., by RFLP analysis, PCR product length(s) distribution, etc.) or for diagnosis of diseases characterized by amplification and/or rearrangements of the NF-AT_c gene.

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Production of NF-AT_c Polypeptides

The nucleotide and amino acid sequences shown in Fig. 1 enable those of skill in the art to produce polypeptides corresponding to all or part of the full-length human NF-AT_c polypeptide sequence. Such polypeptides may be produced in prokaryotic or eukaryotic host cells by expression of polynucleotides encoding NF-AT_c, or fragments and analogs thereof. Alternatively, such polypeptides may be synthesized by chemical methods or produced by *in vitro* translation systems using a polynucleotide template to direct translation. Methods for expression of heterologous proteins in recombinant hosts,

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chemical synthesis of polypeptides, and in vitro translation are well known in the art and are described further in Maniatis et al., Molecular Cloning: A Laboratory Manual (1989), 2nd Ed., Cold Spring Harbor, N.Y. and Berger and Kimmel, Methods in Enzymology, Volume 152, Guide to Molecular Cloning Techniques (1987), Academic Press, Inc., San Diego, CA.

Fragments or analogs of NF-AT_c may be prepared by those of skill in the art. Preferred amino- and carboxy-termini of fragments or analogs of NF-AT_c occur near boundaries of functional domains. For example, but not for limitation, such functional domains include: (1) domains conferring the property of binding to other NF-AT components (e.g., AP-1), (2) domains conferring the property of nuclear localization in stimulated T lymphocytes, and (3) domains conferring the property of enhancing activation of T cells when expressed at sufficient levels in such cells. Additionally, such functional domains might include: (1) domains conferring the property of binding to RNA polymerase species, (2) domains having the capacity to directly alter local chromatin structure, which may comprise catalytic activities (e.g., topoisomerases, endonucleases) and/or which may comprise structural features (e.g., zinc fingers, histone-binding moieties), and (3) domains which may interact with accessory proteins and/or transcription factors.

One method by which structural and functional domains may be identified is by comparison of the nucleotide and/or amino acid sequence data shown in Fig. 1 to public or proprietary sequence databases. Preferably, computerized comparison methods are used to identify sequence motifs or predicted protein conformation domains that occur in other proteins of known structure and/or function, such as the zinc fingers. For example, the NAD-binding domains of dehydrogenases, particularly lactate dehydrogenase and malate dehydrogenase, are similar in conformation and have amino acid sequences that are detectably homologous (Proteins, Structures and Molecular Principles, (1984) Creighton (ed.), W.H. Freeman and Company, New York, which is incorporated herein by reference). Further, a method to identify protein sequences

that fold into a known three-dimensional structure are known (Bowie et al. (1991) Science 253: 164). Thus, the foregoing examples demonstrate that those of skill in the art can recognize sequence motifs and structural conformations that may be used to define structural and functional domains in the NF-AT_c sequences of the invention. One example of a domain is the rel similarity region from amino acid 418 to amino acid 710 of the NF-AT_c polypeptide sequence of Fig. 1.

Additionally, computerized comparison of sequences shown in Fig. 1 to existing sequence databases can identify sequence motifs and structural conformations found in other proteins or coding sequences that indicate similar domains of the NF-AT_c protein. For example but not for limitation, the programs GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package (Genetics Computer Group, 575 Science Dr., Madison, WI) can be used to identify sequences in databases, such as GenBank/EMBL, that have regions of homology with a NF-AT_c sequences. Such homologous regions are candidate structural or functional domains. Alternatively, other algorithms are provided for identifying such domains from sequence data. Further, neural network methods, whether implemented in hardware or software, may be used to: (1) identify related protein sequences and nucleotide sequences, and (2) define structural or functional domains in NF-AT_c polypeptides (Brunak et al. (1991) J. Mol. Biol. 220: 49, which is incorporated herein by reference). For example, the 13-residue repeat motifs -SPRASVTEESWLG- and -SPRVSVTDDSWLG- are examples of structurally related domains.

Fragments or analogs comprising substantially one or more functional domain may be fused to heterologous polypeptide sequences, wherein the resultant fusion protein exhibits the functional property(ies) conferred by the NF-AT_c fragment. Alternatively, NF-AT_c polypeptides wherein one or more functional domain have been deleted will exhibit a loss of the property normally conferred by the missing fragment.

By way of example and not limitation, the domain conferring the property of nuclear localization and/or interaction with AP-1 may be fused to β -galactosidase to

produce a fusion protein that is localized to the nucleus and which can enzymatically convert a chromogenic substrate to a chromophore.

Although one class of preferred embodiments are fragments having amino- and/or carboxy-termini corresponding to amino acid positions near functional domains borders, alternative NF-AT_c fragments may be prepared. The choice of the amino- and carboxy-termini of such fragments rests with the discretion of the practitioner and will be made based on experimental considerations such as ease of construction, stability to proteolysis, thermal stability, immunological reactivity, amino- or carboxyl-terminal residue modification, or other considerations.

In addition to fragments, analogs of NF-AT_c can be made. Such analogs may include one or more deletions or additions of amino acid sequence, either at the amino- or carboxy-termini, or internally, or both; analogs may further include sequence transpositions. Analogues may also comprise amino acid substitutions, preferably conservative substitutions. Additionally, analogs may include heterologous sequences generally linked at the amino- or carboxy-terminus, wherein the heterologous sequence(s) confer a functional property to the resultant analog which is not indigenous to the native NF-AT_c protein. However, NF-AT_c analogs must comprise a segment of 25 amino acids that has substantial similarity to a portion of the amino acid sequence shown in Fig. 1, respectively, and which has at least one of the requisite functional properties enumerated in the Definitions (*supra*). Preferred amino acid substitutions are those which: (1) reduce susceptibility to proteolysis, (2) reduce susceptibility to oxidation, (3) alter post-translational modification of the analog, possibly including phosphorylation, and (4) confer or modify other physicochemical or functional properties of such analogs, possibly including interaction with calcineurin or phosphorylation or dephosphorylation thereby. NF-AT_c analogs include various muteins of a NF-AT_c sequence other than the naturally-occurring peptide sequence. For example, single or multiple amino acid substitutions (preferably conservative

amino acid substitutions) may be made in the naturally-occurring NF-AT_c sequence (preferably in the portion of the polypeptide outside the functional domains).

Conservative amino acid substitution is a substitution of an amino acid by a replacement amino acid which has similar characteristics (e.g., those with acidic properties: Asp and Glu). A conservative (or synonymous) amino acid substitution should not substantially change the structural characteristics of the parent sequence (e.g., a replacement amino acid should not tend to break a helix that occurs in the parent sequence, or disrupt other types of secondary structure that characterizes the parent sequence). Examples of art-recognized polypeptide secondary and tertiary structures are described in Proteins, Structures and Molecular Principles, (1984) Creighton (ed.), W.H. Freeman and Company, New York; Introduction to Protein Structure, (1991), C. Branden and J. Tooze, Garland Publishing, New York, NY; and Thornton et al. (1991) Nature 354: 105; which are incorporated herein by reference).

Native NF-AT_c proteins, fragments thereof, or analogs thereof can be used as reagents in DNA binding assays and/or in vitro transcription assays for identifying agents that interfere with NF-AT function, said agents are thereby identified as candidate drugs which may be used, for example, to block T cell activation or treat T cell lymphocytic leukemias. Typically, in vitro DNA binding assays that measure binding of NF-AT to DNA employ double-stranded DNA that contains an array of one or more NF-AT recognition sites (as defined by specific footprinting of native NF-AT protein). The DNA is typically linked to a solid substrate by any of various means known to those of skill in the art; such linkage may be noncovalent (e.g., binding to a highly charged surface such as Nylon 66) or may be by covalent bonding (e.g., typically by chemical linkage involving a nitrogen position in a nucleotide base, such as diazotization). NF-AT_c polypeptides are typically labeled by incorporation of a radiolabeled amino acid. The labeled NF-AT_c polypeptide, usually reconstituted with an NF-AT nuclear component (e.g., AP-1 activity) to form

an NF-AT complex, is contacted with the immobilized DNA under aqueous conditions that permit specific binding in control binding reactions with a binding affinity of about $1 \times 10^6 \text{ M}^{-1}$ or greater (e.g., 10-250 mM NaCl or KCl and 5-100 mM Tris HCl pH 5-9, usually pH 6-8), generally including Zn^{+2} and/or Mn^{+2} and/or Mg^{+2} in the nanomolar to micromolar range (1 nM to 999 μM). Specificity of binding is typically established by adding unlabeled competitor at various concentrations selected at the discretion of the practitioner. Examples of unlabeled protein competitors include, but are not limited to, the following: unlabeled NF-AT_c polypeptide, bovine serum albumin, and nuclear protein extracts. Binding reactions wherein one or more agents are added are performed in parallel with a control binding reaction that does not include an agent. Agents which inhibit the specific binding of NF-AT_c polypeptides to DNA, as compared to a control reaction, are identified as candidate immunomodulatory drugs. Also, agents which prevent transcriptional modulation by NF-AT in vitro are thereby identified as candidate immunomodulatory drugs.

20

In addition to NF-AT_c polypeptides consisting only of naturally-occurring amino acids, NF-AT_c peptidomimetics are also provided. Peptide analogs are commonly used in the pharmaceutical industry as non-peptide drugs with properties analogous to those of the template peptide. These types of non-peptide compound are termed "peptide mimetics" or "peptidomimetics" (Fauchere, J. (1986) Adv. Drug Res. 15: 29; Veber and Freidinger (1985) TINS p.392; and Evans et al. (1987) J. Med. Chem 30: 1229, which are incorporated herein by reference) and are usually developed with the aid of computerized molecular modeling. Peptide mimetics that are structurally similar to therapeutically useful peptides may be used to produce an equivalent therapeutic or prophylactic effect. Generally, peptidomimetics are structurally similar to a paradigm polypeptide (i.e., a polypeptide that has a biological or pharmacological activity), such as human NF-AT_c, but have one or more peptide linkages optionally replaced by a linkage selected from the group consisting of: $-\text{CH}_2\text{NH}-$, $-\text{CH}_2\text{S}-$,

35

-CH₂-CH₂-, -CH=CH- (cis and trans), -COCH₂-, -CH(OH)CH₂-, and -CH₂SO-, by methods known in the art and further described in the following references: Spatola, A.F. in "Chemistry and Biochemistry of Amino Acids, Peptides, and Proteins," B. Weinstein, eds., Marcel Dekker, New York, p. 267 (1983); Spatola, A.F., *Vega Data* (March 1983), Vol. 1, Issue 3, "Peptide Backbone Modifications" (general review); Morley, J.S., *Trends Pharm Sci* (1980) pp. 463-468 (general review); Hudson, D. et al., *Int J Pept Prot Res* (1979) 14:177-185 (-CH₂NH-, CH₂CH₂-); Spatola, A.F. et al., *Life Sci* (1986) 38:1243-1249 (-CH₂-S); Hann, M.M., *J Chem Soc Perkin Trans I* (1982) 307-314 (-CH-CH-, cis and trans); Almquist, R.G. et al., *J Med Chem* (1980) 23:1392-1398 (-COCH₂-); Jennings-White, C. et al., *Tetrahedron Lett* (1982) 23:2533 (-COCH₂-); Szelke, M. et al., *European Appln. EP 45665* (1982) CA: 97:39405 (1982) (-CH(OH)CH₂-); Holladay, M.W. et al., *Tetrahedron Lett* (1983) 24:4401-4404 (-C(OH)CH₂-); and Hruby, V.J., *Life Sci* (1982) 31:189-199 (-CH₂-S-); each of which is incorporated herein by reference. A particularly preferred non-peptide linkage is -CH₂NH-. Such peptide mimetics may have significant advantages over polypeptide embodiments, including, for example: more economical production, greater chemical stability, enhanced pharmacological properties (half-life, absorption, potency, efficacy, etc.), altered specificity (e.g., a broad-spectrum of biological activities), reduced antigenicity, and others. Labeling of peptidomimetics usually involves covalent attachment of one or more labels, directly or through a spacer (e.g., an amide group), to non-interfering position(s) on the peptidomimetic that are predicted by quantitative structure-activity data and/or molecular modeling. Such non-interfering positions generally are positions that do not form direct contacts with the macromolecules(s) (e.g., immunoglobulin superfamily molecules) to which the peptidomimetic binds to produce the therapeutic effect. Derivatization (e.g., labelling) of peptidomimetics should not substantially interfere with the desired biological or pharmacological activity of the peptidomimetic. Peptidomimetics of NF-AT_c may be used as competitive or noncompetitive agonists or

antagonists of NF-AT_c function. For example, a NF-AT_c peptidomimetic administered to a stimulated T cell containing NF-AT_c and may compete with the naturally-occurring NF-AT_c and reduce NF-AT activity. Alternatively, an NF-AT_c peptidomimetic
5 administered to a T cell lacking NF-AT_c may induce T cell activation or the like.

Systematic substitution of one or more amino acids of a consensus sequence with a D-amino acid of the same type (e.g., D-lysine in place of L-lysine) may be used to generate
10 more stable peptides. In addition, constrained peptides (including cyclized peptides) comprising a consensus sequence or a substantially identical consensus sequence variation may be generated by methods known in the art (Rizo and Gierasch (1992) Ann. Rev. Biochem. 61: 387, incorporated herein by
15 reference); for example, by adding internal cysteine residues capable of forming intramolecular disulfide bridges which cyclize the peptide.

The amino acid sequences of NF-AT_c polypeptides identified herein will enable those of skill in the art to
20 produce polypeptides corresponding to NF-AT_c peptide sequences and sequence variants thereof. Such polypeptides may be produced in prokaryotic or eukaryotic host cells by expression of polynucleotides encoding a NF-AT_c peptide sequence, frequently as part of a larger polypeptide. Alternatively,
25 such peptides may be synthesized by chemical methods. Methods for expression of heterologous proteins in recombinant hosts, chemical synthesis of polypeptides, and in vitro translation are well known in the art and are described further in Maniatis et al., Molecular Cloning: A Laboratory Manual (1989), 2nd Ed.,
30 Cold Spring Harbor, N.Y.; Berger and Kimmel, Methods in Enzymology, Volume 152, Guide to Molecular Cloning Techniques (1987), Academic Press, Inc., San Diego, CA; Merrifield, J. (1969) J. Am. Chem. Soc. 91: 501; Chaiken I.M. (1981) CRC Crit. Rev. Biochem. 11: 255; Kaiser et al. (1989) Science 243: 187;
35 Merrifield, B. (1986) Science 232: 342; Kent, S.B.H. (1988) Ann. Rev. Biochem. 57: 957; and Offord, R.E. (1980) Semisynthetic Proteins, Wiley Publishing, which are incorporated herein by reference).

Production and Applications of α -NF-AT_c Antibodies

Native NF-AT_c proteins, fragments thereof, or analogs thereof, may be used to immunize an animal for the production of specific antibodies. These antibodies may comprise a polyclonal antiserum or may comprise a monoclonal antibody produced by hybridoma cells. For general methods to prepare antibodies, see Antibodies: A Laboratory Manual, (1988) E. Harlow and D. Lane, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, which is incorporated herein by reference.

For example but not for limitation, a recombinantly produced fragment of human NF-AT_c can be injected into a rat along with an adjuvant following immunization protocols known to those of skill in the art so as to generate an immune response. Typically, approximately at least 1-50 μ g of a NF-AT_c fragment or analog is used for the initial immunization, depending upon the length of the polypeptide. Alternatively or in combination with a recombinantly produced NF-AT_c polypeptide, a chemically synthesized peptide having a NF-AT_c sequence (e.g., peptides exemplified in Table II, *infra*) may be used as an immunogen to raise antibodies which bind a NF-AT_c protein, such as the native human NF-AT_c polypeptide having the sequence shown essentially in Fig. 1 or the native human NF-AT_c polypeptide isoform. Immunoglobulins which bind the recombinant fragment with a binding affinity of at least $1 \times 10^7 \text{ M}^{-1}$ can be harvested from the immunized animal as an antiserum, and may be further purified by immunoaffinity chromatography or other means. Additionally, spleen cells are harvested from the immunized animal (typically rat or mouse) and fused to myeloma cells to produce a bank of antibody-secreting hybridoma cells. The bank of hybridomas can be screened for clones that secrete immunoglobulins which bind the recombinantly produced NF-AT_c polypeptide (or chemically synthesized NF-AT_c polypeptide) with an affinity of at least $1 \times 10^6 \text{ M}^{-1}$. Animals other than mice and rats may be used to raise antibodies; for example, goats, rabbits, sheep, and chickens may also be employed to raise antibodies reactive with a NF-AT_c protein. Transgenic mice having the capacity to produce substantially human antibodies also may be immunized

and used for a source of α -NF-AT_c antiserum and/or for making monoclonal-secreting hybridomas.

Bacteriophage antibody display libraries may also be screened for binding to a NF-AT_c polypeptide, such as a full-length human NF-AT_c protein, a NF-AT_c fragment (e.g., a peptide having a sequence shown in Table II, *infra*), or a fusion protein comprising a NF-AT_c polypeptide sequence of at least 14 contiguous amino acids as shown in Fig. 1 or a polypeptide sequence of Table II (*infra*). Combinatorial libraries of antibodies have been generated in bacteriophage lambda expression systems which may be screened as bacteriophage plaques or as colonies of lysogens (Huse et al. (1989) Science 246: 1275; Caton and Koprowski (1990) Proc. Natl. Acad. Sci. (U.S.A.) 87: 6450; Mullinax et al (1990) Proc. Natl. Acad. Sci. (U.S.A.) 87: 8095; Persson et al. (1991) Proc. Natl. Acad. Sci. (U.S.A.) 88: 2432). Various embodiments of bacteriophage antibody display libraries and lambda phage expression libraries have been described (Kang et al. (1991) Proc. Natl. Acad. Sci. (U.S.A.) 88: 4363; Clackson et al. (1991) Nature 352: 624; McCafferty et al. (1990) Nature 348: 552; Burton et al. (1991) Proc. Natl. Acad. Sci. (U.S.A.) 88: 10134; Hoogenboom et al. (1991) Nucleic Acids Res. 19: 4133; Chang et al. (1991) J. Immunol. 147: 3610; Breitling et al. (1991) Gene 104: 147; Marks et al. (1991) J. Mol. Biol. 222: 581; Barbas et al. (1992) Proc. Natl. Acad. Sci. (U.S.A.) 89: 4457; Hawkins and Winter (1992) J. Immunol. 22: 867; Marks et al. (1992) Biotechnology 10: 779; Marks et al. (1992) J. Biol. Chem. 267: 16007; Lowman et al (1991) Biochemistry 30: 10832; Lerner et al. (1992) Science 258: 1313, incorporated herein by reference). Typically, a bacteriophage antibody display library is screened with a NF-AT_c polypeptide that is immobilized (e.g., by covalent linkage to a chromatography resin to enrich for reactive phage by affinity chromatography) and/or labeled (e.g., to screen plaque or colony lifts).

NF-AT_c polypeptides which are useful as immunogens, for diagnostic detection of α -NF-AT_c antibodies in a sample, for diagnostic detection and quantitation of NF-AT_c protein in a sample (e.g., by standardized competitive ELISA), or for

screening a bacteriophage antibody display library, are suitably obtained in substantially pure form, that is, typically about 50 percent (w/w) or more purity, substantially free of interfering proteins and contaminants. Preferably, these polypeptides are isolated or synthesized in a purity of at least 80 percent (w/w) and, more preferably, in at least about 95 percent (w/w) purity, being substantially free of other proteins of humans, mice, or other contaminants. Preferred immunogens comprise at least one NF-AT_c polypeptide sequence shown in Table II, either as a discrete peptide or as part of a fusion polypeptide (e.g., with a β -galactosidase or glutathione S-transferase sequence). NF-AT_c immunogens comprise at least one, typically several of such immunogenic epitopes.

For some applications of these antibodies, such as identifying immunocrossreactive proteins, the desired antiserum or monoclonal antibody(ies) is/are not monospecific. In these instances, it may be preferable to use a synthetic or recombinant fragment of NF-AT_c as an antigen rather than using the entire native protein. More specifically, where the object is to identify immunocrossreactive polypeptides that comprise a particular structural moiety, such as a DNA-binding domain, it is preferable to use as an antigen a fragment corresponding to part or all of a commensurate structural domain in the NF-AT_c protein. Production of recombinant or synthetic fragments having such defined amino- and carboxy-termini is provided by the NF-AT_c sequences shown in Fig. 1.

If an antiserum is raised to a NF-AT_c fusion polypeptide, such as a fusion protein comprising a NF-AT_c immunogenic epitope fused to β -galactosidase or glutathione S-transferase, the antiserum is preferably preadsorbed with the non-NF-AT_c fusion partner (e.g., β -galactosidase or glutathione S-transferase) to deplete the antiserum of antibodies that react (i.e., specifically bind to) the non-NF-AT_c portion of the fusion protein that serves as the immunogen. Monoclonal or polyclonal antibodies which bind to the human and/or murine NF-AT_c protein can be used to detect the presence of human or murine NF-AT_c polypeptides in a sample, such as a Western blot

of denatured protein (e.g., a nitrocellulose blot of an SDS-PAGE) obtained from a lymphocyte sample of a patient. Preferably quantitative detection is performed, such as by denistometric scanning and signal integration of a Western blot. The monoclonal or polyclonal antibodies will bind to the denatured NF-AT_c epitopes and may be identified visually or by other optical means with a labeled second antibody or labeled Staphylococcus aureus protein A by methods known in the art. Frequently, denatured NF-AT_c will be used as the target antigen so that more epitopes may be available for binding.

Table II

Selected Human NF-AT_c Antigen Peptide Sequences

	-NAIFLTVSREHERVGC-;	-AKTDRDLCKPNSLVVEIPFRN-;
15	-LHGYLENEPLMLQLFIGT-;	-EVQPKSHHRAHYETEGSR-;
	-PSTSPRASVTEESWLG-;	-SPRVSVTDDSWLGNT-;
	-GPAPRAGGTMKSAEEHHYG-;	-SHHRAHYETEGSRGAV-;
	-ASAGGHPIVQ-;	-LRNSDIELRKGETDIGR-;
	-NTRVRLVFRV-;	-TSLQVASNPIC-.

Such NF-AT_c sequences as shown in Tables II may be used as an immunogenic peptide directly (e.g., to screen bacteriophage antibody display libraries or to immunize a rabbit), or may be conjugated to a carrier macromolecule (e.g., BSA) or may compose part of a fusion protein to be used as an immunogen. A preferred NF-AT_c polypeptide comprises the following amino acids sequences:

	-PSTSPRASVTEESWLG-;	-SPRVSVTDDSWLGNT-;
	-SHHRAHYETEGSRGAV-;	-NAIFLTVSREHERVGC-;

and may comprise other intervening and/or terminal sequences; generally such polypeptides are less than 1000 amino acids in length, more usually less than about 500 amino acids in length; often spacer peptide sequences or terminal peptide sequences, if present, correspond to naturally occurring polypeptide sequences, generally mammalian polypeptide sequences. One application of the preferred NF-AT_c polypeptide just recited is as a commercial immunogen to raise α -NF-AT_c antibodies in a suitable animal and/or as a commercial immunodiagnostic reagent

for quantitative ELISA (e.g., competitive ELISA) or competitive RIA in conjunction with the anti-NF-AT_c antibodies provided by the invention, such as for calibration of standardization of such immunoassays for staging or diagnosis of NF-AT_c-expressing lymphocytic leukemias in humans or cell typing or identification of T cells (such as activated T cells and/or activatable T cells). The preferred NF-AT_c polypeptide just recited will find many other uses in addition to serving as an immunogen or immunological reagent. One or more of the above-listed sequences may be incorporated into a fusion protein with a fusion partner such as human serum albumin, GST, etc. For such fusion proteins in excess of 1000 amino acids, deletions in the fusion partner (albumin) moiety may be made to bring the size to about 1000 amino acids or less, if desired.

In some embodiments, it will be desirable to employ a polyvalent NF-AT_c antigen, comprising at least two NF-AT_c immunogenic epitopes in covalent linkage, usually in peptide linkage. Such polyvalent NF-AT_c antigens typically comprise multiple NF-AT_c antigenic peptides from the same species (e.g., human or mouse), but may comprise a mix of antigenic peptides from NF-AT_c proteins of different species (i.e., an interspecies NF-AT_c polyvalent antigen). Frequently, the spatial order of the antigenic peptide sequences in the primary amino acid sequence of a polyvalent antigen occurs in the same orientation as in the naturally occurring NF-AT_c protein (i.e., a first antigenic peptide sequence that is amino-terminal to a second antigenic peptide sequence in a naturally occurring NF-AT_c protein will be amino-terminal to said second antigenic peptide sequence in a polyvalent antigen. Frequently, spacer peptide sequences will be used to link antigenic peptide sequences in a polyvalent antigen, such spacer peptide sequences may be predetermined, random, or pseudorandom sequences. Spacer peptide sequences may correspond to sequences known to be non-immunogenic to the animal which is to be immunized with the polyvalent antigen, such as a sequence to which the animal has been tolerized. Although many examples of such polyvalent antigens may be given, the following embodiment is provided for illustration and not limitation:

-NAIFLTVSREHERVGC-(aa1)-AKTDRDLCKPNSLVVEIPFRN-(aa2)-
VKASAGGHPIVQL

5 where (aa1) and (aa2) are peptide spacers of at least one amino acid and less than 1000 amino acids; aa1 is a peptide sequence selected independently from the aa2 peptide sequence; the length of aa1 (which may be composed of multiple different amino acids) is independent of the length of aa2 (which may be composed of multiple different amino acids).

10 Immunogenic NF-AT_c peptides may be used to immunize an animal to raise anti-NF-AT_c antibodies and/or as a source of spleen cells for making a hybridoma library from which to select hybridoma clones which secrete a monoclonal antibody which binds to a NF-AT_c protein with an affinity of $1 \times 10^7 \text{ M}^{-1}$
15 or greater, preferably at least $1 \times 10^8 \text{ M}^{-1}$ to $1 \times 10^9 \text{ M}^{-1}$. Such immunogenic NF-AT_c peptides can also be used to screen bacteriophage antibody display libraries directly.

One use of such antibodies is to screen cDNA expression libraries, preferably containing cDNA derived from
20 human or murine mRNA from various tissues, for identifying clones containing cDNA inserts which encode structurally-related, immunocrossreactive proteins, that are candidate novel transcription factors or chromatin proteins. Such screening of cDNA expression libraries is well known in the art, and is
25 further described in Young et al., Proc. Natl. Acad. Sci. U.S.A. 80:1194-1198 (1983), which is incorporated herein by reference] as well as other published sources. Another use of such antibodies is to identify and/or purify immunocrossreactive proteins that are structurally or
30 evolutionarily related to the native NF-AT_c protein or to the corresponding NF-AT_c fragment (e.g., functional domain; DNA-binding domain) used to generate the antibody. It is believed that such antibodies will find commercial use as such reagents for research applications, just as other antibodies (and
35 biological reagents - such as restriction enzymes and polymerases) are sold commercially.

Various other uses of such antibodies are to diagnose and/or stage leukemias or other immunological disease states,

and for therapeutic application (e.g., as cationized antibodies or by targeted liposomal delivery) to treat neoplasia, hyperimmune function, graft rejection, and the like.

An example of an NF-AT_c polypeptide is a polypeptide
 5 having the sequence:
 MPSTSFPVPSKFPPLGPAAVFGRGETLGPAPRAGGTMKSAEEEHYGYASSNVSPALPLPTAHS
 TLPAPCHNLQTSTPGIIPADHPSGYGAALDGCPAGYFLSSGHTRPDGAPALESPRIEITSCL
 GLYHNNNQFFHDVEVEDVLPSSKRSPSTATLSLPSLEAYRDPSCLSPASSLSSRSCNSEASSY
 ESNYSYPYASPQTSPWQSPCVSPKTTDPEEGFPRGLGACTLLGSPQHSPSTSPRASVTEESWL
 10 GARSSRPASPCNKRYSLNGRQPPYPSPHHSPTSPHGSPPRVSVTDDSWLGNTTQYTSSAIVAA
 INALTTDSSLDLGDGVPVKSRKTTLEQPPSVALKVEPVGEDLGSPPPPADFPEDYSSFOHIR
 KGGFCDQYLAVPQHPYQWAKPKPLSPTSYSMSPTLPALDWQLPSHSGPYELRIEVQPKSHHRAH
 YETEGSRGAVKASAGGHPVQLHGYLENEPLMLQLFIGTADDRLLRPAPFYQVHRITGKTVST
 TSHEAILSNTKVLEIPLLPENSMRAVIDCACILKLRNSDIELRKGETDIGRKNTRVRLVFRVH
 15 VPQPSGRTLSQLQVASNPIECSQRSQAEPLVEKQSTDSYPVVGKKMVLSGHNFLQDSKVIFV
 EKAPDGHVWEMEAKTDRDLCKPNSLVVEIPFRNQIRITSPVHVSFYVCNGKRKRSQYQRTY
 LPANGNAIFLTVSREHERVGCFF

NF-AT_c Polynucleotides

20 Disclosure of the full coding sequence for human NF-AT_c shown in Fig. 1 makes possible the construction of isolated polynucleotides that can direct the expression of NF-AT_c, fragments thereof, or analogs thereof. Further, the sequences in Fig. 1 make possible the construction of nucleic acid
 25 hybridization probes and PCR primers that can be used to detect RNA and DNA sequences encoding NF-AT_c.

Polynucleotides encoding full-length NF-AT_c or fragments or analogs thereof, may include sequences that facilitate transcription (expression sequences) and translation
 30 of the coding sequences, such that the encoded polypeptide product is produced. Construction of such polynucleotides is well known in the art and is described further in Maniatis et al., Molecular Cloning: A Laboratory Manual, 2nd Ed. (1989), Cold Spring Harbor, N.Y. For example, but not for limitation,
 35 such polynucleotides can include a promoter, a transcription termination site (polyadenylation site in eukaryotic expression hosts), a ribosome binding site, and, optionally, an enhancer for use in eukaryotic expression hosts, and, optionally,

sequences necessary for replication of a vector. A typical eukaryotic expression cassette will include a polynucleotide sequence encoding a NF-AT_c polypeptide linked downstream (i.e., in translational reading frame orientation; polynucleotide linkage) of a promoter such as the HSV tk promoter or the *pgk* (phosphoglycerate kinase) promoter, optionally linked to an enhancer and a downstream polyadenylation site (e.g., an SV40 large T Ag poly A addition site).

A preferred NF-AT_c polynucleotide encodes a NF-AT_c polypeptide that comprises at least one of the following amino acids sequences:

-NAIFLTVSREHERVGC-;	-AKTDRDLCKPNSLVVEIPFRN-;
-LHGYLENEPLMLQLFIGT-;	-EVQPKSHHRAHYETEGSR-;
-PSTSPRASVTEESWLG-;	-SPRVSVTDDSWLGNT-;
-GPAPRAGGTMKSAEEEHYG-;	-SHHRAHYETEGSRGAV-;
-ASAGGHPIVQ-;	-LRNSDIELRKGETDIGR-;
-NTRVRLVFRV-;	-TSLQVASNPIC-

A preferred NF-AT_c polynucleotide encodes a NF-AT_c polypeptide that comprises two, three, four, five, six, seven, eight, nine, ten, eleven, or all twelve of the following amino acids sequences:

-NAIFLTVSREHERVGC-;	-AKTDRDLCKPNSLVVEIPFRN-;
-LHGYLENEPLMLQLFIGT-;	-EVQPKSHHRAHYETEGSR-;
-PSTSPRASVTEESWLG-;	-SPRVSVTDDSWLGNT-;
-GPAPRAGGTMKSAEEEHYG-;	-SHHRAHYETEGSRGAV-;
-ASAGGHPIVQ-;	-LRNSDIELRKGETDIGR-;
-NTRVRLVFRV-;	-TSLQVASNPIC-

The degeneracy of the genetic code gives a finite set of polynucleotide sequences encoding these amino acid sequences; this set of degenerate sequences may be readily generated by hand or by computer using commercially available software (Wisconsin Genetics Software Package Release 7.0). Thus, isolated polynucleotides typically less than approximately 10,000 nucleotides in length, often less than 3000 nucleotides in length and comprising sequences encoding each of the following amino acid sequences:

-NAIFLTVSREHERVGC-;	-AKTDRDLCKPNSLVVEIPFRN-;
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-LHGYLENEPLMLQLFIGT-;	-EVQPKSHHRAHYETEGSR-;
-PSTSPRASVTEESWLG-;	-SPRVSVTDDSWLGNT-;
-GPAPRAGGTMKSAEEEEHYG-;	-SHHRAHYETEGSRGAV-;
-ASAGGHPIVQ-;	-LRNSDIELRKGETDIGR-;
-NTRVRLVFRV-;	-TSLQVASNPIC-

are provided and may be used for, among other uses, the expression of a NF-AT_c polypeptide which can be used as an immunogen, immunological reagent, and the like. Such polynucleotides typically comprise an operably linked promoter for driving expression in a suitable prokaryotic or eukaryotic host cell. One exemplification of such a polynucleotide is the human NF-AT_c cDNA sequence of Fig. 1 cloned in operable linkage to the mammalian expression vector pSR α , many alternative embodiments will be apparent to those of skill in the art, including the use of alternative expression vectors (e.g., pBC12BI and p91023(B); Hanahan J (1983) J. Mol. Biol. 166: 577; Cullen et al. (1985) J. Virol. 53: 515; Lomedico PT (1982) Proc. Natl. Acad. Sci. (U.S.A.) 79: 5798; Morinaga et al. (1984) Bio/Technology 2: 636).

Additionally, where expression of a polypeptide is not desired, polynucleotides of this invention need not encode a functional protein. Polynucleotides of this invention may serve as hybridization probes and/or PCR primers (amplimers) and/or LCR oligomers for detecting NF-AT_c RNA or DNA sequences.

Alternatively, polynucleotides of this invention may serve as hybridization probes or primers for detecting RNA or DNA sequences of related genes, such genes may encode structurally or evolutionarily related proteins. For such hybridization and PCR applications, the polynucleotides of the invention need not encode a functional polypeptide. Thus, polynucleotides of the invention may contain substantial deletions, additions, nucleotide substitutions and/or transpositions, so long as specific hybridization or specific amplification to the NF-AT_c sequence is retained.

Specific hybridization is defined hereinbefore, and can be roughly summarized as the formation of hybrids between a polynucleotide of the invention (which may include substitutions, deletions, and/or additions) and a specific

target polynucleotide such as human NF-AT_c mRNA so that a single band is identified corresponding to each NF-AT_c isoform on a Northern blot of RNA prepared from T cells (i.e., hybridization and washing conditions can be established that permit detection of discrete NF-AT_c mRNA band(s)). Thus, those of ordinary skill in the art can prepare polynucleotides of the invention, which may include substantial additions, deletions, substitutions, or transpositions of nucleotide sequence as compared to sequences shown in Fig. 1 and determine whether specific hybridization is a property of the polynucleotide by performing a Northern blot using RNA prepared from a T lymphocyte cell line which expresses NF-AT_c mRNA and/or by hybridization to a NF-AT_c DNA clone (cDNA or genomic clone).

Specific amplification is defined as the ability of a set of PCR amplimers, when used together in a PCR reaction with a NF-AT_c polynucleotide, to produce substantially a single major amplification product which corresponds to a NF-AT_c gene sequence or mRNA sequence. Generally, human genomic DNA or mRNA from NF-AT_c expressing human cells (e.g., Jurkat cell line) is used as the template DNA sample for the PCR reaction. PCR amplimers that exhibit specific amplification are suitable for quantitative determination of NF-AT_c mRNA by quantitative PCR amplification. NF-AT_c allele-specific amplification products, although having sequence and/or length polymorphisms, are considered to constitute a single amplification product for purposes of this definition.

Generally, hybridization probes comprise approximately at least 25 consecutive nucleotides of a sequence shown in Fig. 1 (for human and murine NF-AT_c detection, respectively), preferably the hybridization probes contain at least 50 consecutive nucleotides of a sequence shown in Fig. 1, and more preferably comprise at least 100 consecutive nucleotides of a sequence shown in Fig. 1. PCR amplimers typically comprise approximately 25 to 50 consecutive nucleotides of a sequence shown in Fig. 1, and usually consist essentially of approximately 25 to 50 consecutive nucleotides of a sequence shown in Fig. 1 with additional nucleotides, if present, generally being at the 5' end so as not to interfere

with polymerase-mediated chain extension. PCR amplifier design and hybridization probe selection are well within the scope of discretion of practitioners of ordinary skill in the art.

5 Methods Relating to Genetic Disease

In one preferred embodiment of the invention, hybridization probes that specifically identify the NF-AT_c gene may be used in methods for diagnosing genetic disease. For example, but not for limitation, the genetic disease thus
10 diagnosed may involve a lesion in the relevant NF-AT_c structural or regulatory sequences, or may involve a lesion in a genetic locus closely linked to the NF-AT_c locus and which can be identified by restriction fragment length polymorphism or DNA sequence polymorphism at the linked NF-AT_c locus. In a
15 further preferred embodiment, NF-AT_c gene probes are used to diagnose or identify genetic disease involving predisposition to immunological disease, wherein the amount or functionality of endogenous NF-AT_c is sufficient for the individual to exhibit an increased probability of developing an immune
20 disease, particularly an immune deficiency, arthritis, or autoimmune disease.

Antisense Polynucleotides

Additional embodiments directed to modulation of T
25 cell activation include methods that employ specific antisense polynucleotides complementary to all or part of the sequences shown in Fig. 1. Such complementary antisense polynucleotides may include nucleotide substitutions, additions, deletions, or transpositions, so long as specific hybridization to the
30 relevant target sequence corresponding to Fig. 1 is retained as a functional property of the polynucleotide. Complementary antisense polynucleotides include soluble antisense RNA or DNA oligonucleotides which can hybridize specifically to NF-AT_c mRNA species and prevent transcription of the mRNA species
35 and/or translation of the encoded polypeptide (Ching et al. (1989) Proc. Natl. Acad. Sci. U.S.A. 86: 10006; Broder et al. (1990) Ann. Int. Med. 113: 604; Loreau et al. (1990) FEBS Letters 274: 53; Holcenberg et al., WO91/11535; U.S.S.N.

07/530,165; WO91/09865; WO91/04753; WO90/13641; and EP 386563, each of which is incorporated herein by reference). The antisense polynucleotides therefore inhibit production of NF-AT_c polypeptides. Since NF-AT_c protein expression is associated with T lymphocyte activation, antisense polynucleotides that prevent transcription and/or translation of mRNA corresponding to NF-AT_c polypeptides may inhibit T cell activation and/or reverse the the activated phenotype of T cells. Compositions containing a therapeutically effective dosage of NF-AT_c antisense polynucleotides may be administered for treatment of immune diseases, including lymphocytic leukemias, and for inhibition of transplant rejection reactions, if desired. Antisense polynucleotides of various lengths may be produced, although such antisense polynucleotides typically comprise a sequence of about at least 25 consecutive nucleotides which are substantially identical to a naturally-occurring NF-AT_c polynucleotide sequence, and typically which are identical to a sequence shown in Fig. 1.

Antisense polynucleotides may be produced from a heterologous expression cassette in a transfectant cell or transgenic cell, such as a transgenic pluripotent hematopoietic stem cell used to reconstitute all or part of the hematopoietic stem cell population of an individual. Alternatively, the antisense polynucleotides may comprise soluble oligonucleotides that are administered to the external milieu, either in the culture medium in vitro or in the circulatory system or interstitial fluid in vivo. Soluble antisense polynucleotides present in the external milieu have been shown to gain access to the cytoplasm and inhibit translation of specific mRNA species. In some embodiments the antisense polynucleotides comprise methylphosphonate moieties or are polyamide nucleic acids (PNAs). For general methods relating to antisense polynucleotides, see Antisense RNA and DNA, (1988), D.A. Melton, Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

Isolation of the Cognate Human NF-AT_c Gene

The human homolog of the NF-AT_c cDNA is identified and isolated by screening a human genomic clone library, such as a human genomic library in yeast artificial chromosomes, cosmids, or bacteriophage λ (e.g., λ Charon 35), with a polynucleotide probe comprising a sequence of about at least 24 contiguous nucleotides (or their complement) of the cDNA sequence shown in Fig. 1. Typically, hybridization and washing conditions are performed at high stringency according to conventional hybridization procedures. Positive clones are isolated and sequenced. For illustration and not for limitation, a full-length polynucleotide corresponding to the sequence of Fig. 1 may be labeled and used as a hybridization probe to isolate genomic clones from a human or murine genomic clone library in λ EMBL4 or λ GEM11 (Promega Corporation, Madison, Wisconsin); typical hybridization conditions for screening plaque lifts (Benton and Davis (1978) Science 196: 180) can be: 50% formamide, 5 x SSC or SSPE, 1-5 x Denhardt's solution, 0.1-1% SDS, 100-200 μ g sheared heterologous DNA or tRNA, 0-10% dextran sulfate, 1×10^5 to 1×10^7 cpm/ml of denatured probe with a specific activity of about 1×10^8 cpm/ μ g, and incubation at 42°C for about 6-36 hours. Prehybridization conditions are essentially identical except that probe is not included and incubation time is typically reduced. Washing conditions are typically 1-3 x SSC, 0.1-1% SDS, 50-70°C with change of wash solution at about 5-30 minutes.

Nonhuman NF-AT_c cDNAs and genomic clones (i.e., cognate nonhuman NF-AT_c genes) can be analogously isolated from various nonhuman cDNA and genomic clone libraries available in the art (e.g., Clontech, Palo Alto, CA) by using probes based on the sequences shown in Fig. 1, with hybridization and washing conditions typically being less stringent than for isolation of human NF-AT_c clones.

Polynucleotides comprising sequences of approximately at least 30-50 nucleotides, preferably at least 100 nucleotides, corresponding to or complementary to the nucleotide sequences shown in Fig. 1 can serve as PCR primers and/or hybridization probes for identifying and isolating germline genes corresponding to NF-AT_c. These germline genes

- may be human or may be from a related mammalian species, preferably rodents or primates. Such germline genes may be isolated by various methods conventional in the art, including, but not limited to, by hybridization screening of genomic libraries in bacteriophage λ or cosmid libraries, or by PCR amplification of genomic sequences using primers derived from the sequences shown in Fig. 1. Human genomic libraries are publicly available or may be constructed de novo from human DNA.
- 10 Genomic clones of NF-AT_c, particularly of the murine cognate NF-AT_c gene, may be used to construct homologous targeting constructs for generating cells and transgenic nonhuman animals having at least one functionally disrupted NF-AT_c allele, preferably homozygous for knocked out NF-AT_c alleles. Guidance for construction of homologous targeting constructs may be found in the art, including: Rahemtulla et al. (1991) Nature 353: 180; Jasin et al. (1990) Genes Devel. 4: 157; Koh et al. (1992) Science 256: 1210; Molina et al. (1992) Nature 357: 161; Grusby et al. (1991) Science 253: 1417; 20 Bradley et al. (1992) Bio/Technology 10: 534, incorporated herein by reference). Homologous targeting can be used to generate so-called "knockout" mice, which are heterozygous or homozygous for an inactivated NF-AT_c allele. Such mice may be sold commercially as research animals for investigation of 25 immune system development, neoplasia, T cell activation, signal transduction, drug screening, and other uses.
- Chimeric targeted mice are derived according to Hogan, et al., Manipulating the Mouse Embryo: A Laboratory Manual, Cold Spring Harbor Laboratory (1988) and 30 Teratocarcinomas and Embryonic Stem Cells: A Practical Approach, E.J. Robertson, ed., IRL Press, Washington, D.C., (1987) which are incorporated herein by reference. Embryonic stem cells are manipulated according to published procedures (Teratocarcinomas and Embryonic Stem Cells: A 35 Practical Approach, E.J. Robertson, ed., IRL Press, Washington, D.C. (1987); Zijlstra et al. (1989) Nature 342:435; and Schwartzberg et al. (1989) Science 246: 799, each of which is incorporated herein by reference).

Additionally, a NF-AT_c cDNA or genomic gene copy may be used to construct transgenes for expressing NF-AT_c polypeptides at high levels and/or under the transcriptional control of transcription control sequences which do not naturally occur adjacent to the NF-AT_c gene. For example but not limitation, a constitutive promoter (e.g., a HSV-tk or pgk promoter) or a cell-lineage specific transcriptional regulatory sequence (e.g., a CD4 or CD8 gene promoter/enhancer) may be operably linked to a NF-AT_c-encoding polynucleotide sequence to form a transgene (typically in combination with a selectable marker such as a neo gene expression cassette). Such transgenes can be introduced into cells (e.g., ES cells, hematopoietic stem cells) and transgenic cells and transgenic nonhuman animals may be obtained according to conventional methods. Transgenic cells and/or transgenic nonhuman animals may be used to screen for antineoplastic agents and/or to screen for potential immunomodulatory agents, as overexpression of NF-AT_c or inappropriate expression of NF-AT_c may result in a hyperimmune state or enhance graft rejection reactions.

20

Identification and Isolation of Proteins That Bind NF-AT_c

Proteins that bind to NF-AT_c and/or a NFAT-DNA complex are potentially important transcriptional regulatory proteins. Such proteins may be targets for novel immunomodulatory agents. These proteins are referred to herein as accessory proteins. Accessory proteins may be isolated by various methods known in the art.

One preferred method of isolating accessory proteins is by contacting a NF-AT_c polypeptide to an antibody that binds the NF-AT_c polypeptide, and isolating resultant immune complexes. These immune complexes may contain accessory proteins bound to the NF-AT_c polypeptide. The accessory proteins may be identified and isolated by denaturing the immune complexes with a denaturing agent and, preferably, a reducing agent. The denatured, and preferably reduced, proteins can be electrophoresed on a polyacrylamide gel. Putative accessory proteins can be identified on the polyacrylamide gel by one or more of various well known methods

(e.g., Coomassie staining, Western blotting, silver staining, etc.), and isolated by resection of a portion of the polyacrylamide gel containing the relevant identified polypeptide and elution of the polypeptide from the gel portion.

5 A putative accessory protein may be identified as an accessory protein by demonstration that the protein binds to NF-AT_c and/or a NFAT-DNA complex. Such binding may be shown in vitro by various means, including, but not limited to, binding assays employing a putative accessory protein that has been renatured subsequent to isolation by a polyacrylamide gel electrophoresis method. Alternatively, binding assays employing recombinant or chemically synthesized putative accessory protein may be used. For example, a putative accessory protein may be isolated and all or part of its amino acid sequence determined by chemical sequencing, such as Edman degradation. The amino acid sequence information may be used to chemically synthesize the putative accessory protein. The amino acid sequence may also be used to produce a recombinant putative accessory protein by: (1) isolating a cDNA clone encoding the putative accessory protein by screening a cDNA library with degenerate oligonucleotide probes according to the amino acid sequence data, (2) expressing the cDNA in a host cell, and (3) isolating the putative accessory protein. Alternatively, a polynucleotide encoding a NF-AT_c polypeptide may be constructed by oligonucleotide synthesis, placed in an expression vector, and expressed in a host cell.

Putative accessory proteins that bind NF-AT_c and/or NFAT-DNA complex in vitro are identified as accessory proteins. Accessory proteins may also be identified by crosslinking in vivo with bifunctional crosslinking reagents (e.g., dimethylsuberimidate, glutaraldehyde, etc.) and subsequent isolation of crosslinked products that include a NF-AT_c polypeptide. For a general discussion of cross-linking, see Kunkel et al. (1981) Mol. Cell. Biochem. 34: 3, which is incorporated herein by reference. Preferably, the bifunctional crosslinking reagent will produce crosslinks which may be reversed under specific conditions after isolation of the

crosslinked complex so as to facilitate isolation of the accessory protein from the NF-AT_c polypeptide. Isolation of crosslinked complexes that include a NF-AT_c polypeptide is preferably accomplished by binding an antibody that binds a NF-AT_c polypeptide with an affinity of at least $1 \times 10^7 \text{ M}^{-1}$ to a population of crosslinked complexes and recovering only those complexes that bind to the antibody with an affinity of at least $1 \times 10^7 \text{ M}^{-1}$. Polypeptides that are crosslinked to a NF-AT_c polypeptide are identified as accessory proteins.

Screening assays can be developed for identifying candidate immunomodulatory agents as being agents which inhibit binding of NF-AT_c to an accessory protein (e.g. AP-1) under suitable binding conditions.

Expression of NF-AT_c Polypeptides

The nucleic acid sequences of the present invention capable of ultimately expressing the desired NF-AT_c polypeptides can be formed from a variety of different polynucleotides (genomic or cDNA, RNA, synthetic oligonucleotides, etc.) as well as by a variety of different techniques.

As stated previously, the DNA sequences will be expressed in hosts after the sequences have been operably linked to (*i.e.*, positioned to ensure the functioning of) an expression control sequence. These expression vectors are typically replicable in the host organisms either as episomes or as an integral part of the host chromosomal DNA. Commonly, expression vectors will contain selection markers, *e.g.*, tetracycline resistance or hygromycin resistance, to permit detection and/or selection of those cells transformed with the desired DNA sequences (*see, e.g.*, U.S. Patent 4,704,362, which is incorporated herein by reference).

E. coli is one prokaryotic host useful particularly for cloning the DNA sequences of the present invention. Other microbial hosts suitable for use include bacilli, such as *Bacillus subtilis*, and other Enterobacteriaceae, such as *Salmonella*, *Serratia*, and various *Pseudomonas* species. In these prokaryotic hosts, one can also make expression vectors,

which will typically contain expression control sequences compatible with the host cell (e.g., an origin of replication). In addition, any number of a variety of well-known promoters will be present, such as the lactose promoter system, a
5 tryptophan (trp) promoter system, a beta-lactamase promoter system, or a promoter system from phage lambda. The promoters will typically control expression, optionally with an operator sequence, and have ribosome binding site sequences and the like, for initiating and completing transcription and
10 translation.

Other microbes, such as yeast, may also be used for expression. Saccharomyces is a preferred host, with suitable vectors having expression control sequences, such as promoters, including 3-phosphoglycerate kinase or other glycolytic
15 enzymes, and an origin of replication, termination sequences and the like as desired.

In addition to microorganisms, mammalian tissue cell culture may also be used to express and produce the polypeptides of the present invention (see, Winnacker, "From
20 Genes to Clones," VCH Publishers, N.Y., N.Y. (1987), which is incorporated herein by reference). Eukaryotic cells are actually preferred, because a number of suitable host cell lines capable of secreting intact human proteins have been developed in the art, and include the CHO cell lines, various
25 COS cell lines, HeLa cells, myeloma cell lines, Jurkat cells, etc. Expression vectors for these cells can include expression control sequences, such as an origin of replication, a promoter, an enhancer (Queen et al. (1986) Immunol. Rev. 89: 49, which is incorporated herein by reference), and necessary
30 processing information sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites, and transcriptional terminator sequences. Preferred expression control sequences are promoters derived from immunoglobulin genes, SV40, adenovirus, bovine papillomavirus, and the like. The vectors
35 containing the DNA segments of interest (e.g., polypeptides encoding a NF-AT_c polypeptide) can be transferred into the host cell by well-known methods, which vary depending on the type of cellular host. For example, CaCl transfection is commonly

utilized for prokaryotic cells, whereas CaPO_4 treatment or electroporation may be used for other cellular hosts. (See, generally, Maniatis, et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, (1982), which is incorporated
5 herein by reference). Usually, vectors are episomes and are maintained extrachromosomally.

Expression of recombinant NF-AT_c protein in cells, particularly cells of the lymphopoietic lineage, may be used to identify and isolate genes that are transcriptionally
10 modulated, either positively or negatively, by the presence of NF-AT_c protein. Such genes are typically initially identified as cDNA clones isolated from subtractive cDNA libraries, wherein RNA isolated from cells expressing recombinant NF-AT_c and RNA isolated from control cells (i.e., not expressing
15 recombinant NF-AT_c) are used to generate the subtractive libraries and screening probes. In such a manner, NF-AT_c-dependent genes may be isolated. NFAT-dependent genes (or their regulatory sequences operably linked to a reporter gene) may be used as a component of an in vitro transcription assay
20 employing a NF-AT_c polypeptide as a necessary component for efficient transcription; such transcription assays may be used to screen for agents which inhibit NF-AT_c-dependent gene transcription and are thereby identified as candidate immunomodulatory agents.

25

Methods for Forensic Identification

The NF-AT_c polynucleotide sequences of the present invention can be used for forensic identification of individual humans, such as for identification of decedents, determination
30 of paternity, criminal identification, and the like. For example but not limitation, a DNA sample can be obtained from a person or from a cellular sample (e.g., crime scene evidence such as blood, saliva, semen, and the like) and subjected to RFLP analysis, allele-specific PCR, PCR cloning and sequencing
35 of the amplification product to determine the structure of the NF-AT_c gene region, or other suitable forensic method. On the basis of the NF-AT_c gene structure, the individual from which the sample originated will be identified with respect to

his/her NF-AT_c genotype. The NF-AT_c genotype may be used alone or in conjunction with other genetic markers to conclusively identify an individual or to rule out the individual as a possible perpetrator.

5 In one embodiment, human genomic DNA samples from a population of individuals (typically at least 50 persons from various racial origins) are individually aliquoted into reaction vessels (e.g., a well on a microtitre plate). Each aliquot is digested (incubated) with one or more restriction
10 enzymes (e.g., EcoRI, HindIII, SmaI, BamHI, SalI, NotI, AccI, ApaI, BglII, XbaI, PstI) under suitable reaction conditions (e.g., see New England Biolabs 1992 catalog). Corresponding digestion products from each individual are loaded separately on an electrophoretic gel (typically agarose), electrophoresed,
15 blotted to a membrane by Southern blotting, and hybridized with a labeled NF-AT_c probe (e.g., a full-length human NF-AT_c cDNA sequence of Fig. 1). Restriction fragments (bands) which are polymorphic among members of the population are used as a basis to discriminate NF-AT_c genotypes and thereby classify
20 individuals on the basis of their NF-AT_c genotype.

 Similar categorization of NF-AT_c genotypes may be performed by sequencing PCR amplification products from a population of individuals and using sequence polymorphisms to identify alleles (genotypes), and thereby identify or classify
25 individuals.

Yeast Two-Hybrid Screening Assays

 Yeast two-hybrid systems may be used to screen a mammalian (typically human) cDNA expression library, wherein
30 cDNA is fused to a GAL4 DNA binding domain or activator domain, and a NF-AT_c polypeptide sequence is fused to a GAL4 activator domain or DNA binding domain, respectively. Such a yeast two-hybrid system can screen for cDNAs encoding proteins which bind to NF-AT_c sequences. For example, a cDNA library can be
35 produced from mRNA from a human mature T cell line or other suitable cell type. Such a cDNA library cloned in a yeast two-hybrid expression system (Chien et al. (1991) Proc. Natl. Acad. Sci. (U.S.A.) 88: 9578 or Cell 72: 233) can be used to identify

cdNAs which encode proteins that interact with NF-AT_c and thereby produce expression of the GAL4-dependent reporter gene. Polypeptides which interact with NF-AT_c can also be identified by immunoprecipitation of NF-AT_c with antibody and
5 identification of co-precipitating species. Further, polypeptides that bind NF-AT_c can be identified by screening a peptide library (e.g., a bacteriophage peptide display library, a spatially defined VLSIPS peptide array, and the like) with a NF-AT_c polypeptide.

10

Methods for Rational Drug Design

NF-AT_c polypeptides, especially those portions which form direct contacts in NF-AT complexes, can be used for rational drug design of candidate NFAT-modulating agents (e.g.,
15 antineoplastics and immunomodulators). The substantially purified NF-AT_c and the identification of NF-AT_c as a docking partner for AP-1 activities as provided herein permits production of substantially pure NFAT polypeptide complexes and computational models which can be used for protein X-ray
20 crystallography or other structure analysis methods, such as the DOCK program (Kuntz et al. (1982) J. Mol. Biol. 161: 269; Kuntz ID (1992) Science 257: 1078) and variants thereof. Potential therapeutic drugs may be designed rationally on the basis of structural information thus provided. In one
25 embodiment, such drugs are designed to prevent formation of a NF-AT_c polypeptide: AP-1 polypeptide complex. Thus, the present invention may be used to design drugs, including drugs with a capacity to inhibit binding of NF-AT_c to form NFAT.

Particularly preferred variants are structural
30 mimetics of a dominant negative NF-AT_c mutants, such as a polypeptide consisting essentially of amino acids 1-418 of Fig. 1 and substantially lacking amino acids carboxy-terminal to residue 418. Such mimetics of dominant-negative mutant polypeptides can have substantial activity as antagonists or
35 partial agonists of NF-AT activation (and hence T cell activation).

The following examples are offered by way of example and not by way of limitation. Variations and alternate embodiments will be apparent to those of skill in the art.

EXPERIMENTAL EXAMPLESOVERVIEW

We have purified two related proteins encoded by separate genes that represent the preexisting or cytosolic components of NF-AT. Expression of a full length cDNA for one of these proteins, NF-AT_c, activates the IL-2 promoter in non-T lymphocytes, while a dominant negative of NF-AT_c specifically blocks activation of the IL-2 promoter in T lymphocytes, indicating that NF-AT_c is required for IL-2 gene expression and is responsible for the restricted expression of IL-2. NF-AT_c RNA expression is largely restricted to lymphoid tissues and is induced upon cell activation. The second protein, NF-AT_p, is highly homologous to NF-AT_c over a limited domain, but exhibits wider tissue distribution and is highly expressed in tissues characterized by Ca⁺⁺-dependent regulation. Together these proteins are members of a new family of DNA binding proteins, which are distantly related to the Dorsal/Rel family (Nolan and Baltimore (1992) Current Biology, Ltd. 2: 211-220). Agents that increase intracellular Ca⁺⁺ or that activate protein kinase C independently produce alterations in the mobility of NF-AT_c, indicating that distinct signaling pathways converge on NF-AT_c to regulate its function.

Since our previous work indicated that the cytosolic component of NF-AT was present at relatively low concentrations in human lymphoid cell lines (Northrop et al. (1993) J. Biol. Chem. 268: 2917-2923), we chose to purify NF-AT_c from bovine thymus. Amino acid sequence, obtained from 6 peptides, was used to isolate two overlapping human cDNA clones spanning 2742 nucleotides (Fig. 1). The cDNA encodes a protein of 716 amino acids with a predicted molecular weight of 77,870. An in-frame stop codon upstream from the initiator methionine indicates that the entire NF-AT_c protein is encoded by this cDNA. A unique repeated sequence of 13 residues was also identified. The carboxy-terminal half of NF-AT_c shows limited similarity to the DNA binding and dimerization regions of the Dorsal/Rel family of transcription factors (Fig. 4, for review, Nolan and Baltimore (1992) Current Biology, Ltd. 2: 211-220) however, NF-AT_c appears to be the most distantly related member of the

group. There are a significant number of amino acid changes resulting in charge reversals between the Rel family members and NF-AT_C, suggesting that charge might be conserved at these positions to maintain salt bridges. Six additional peptides
5 obtained from the purified bovine protein are derived from the bovine homolog of NF-AT_P, a cDNA fragment of which was reported by McCaffrey et al. (1993) Science 262: 750-754). Comparison of NF-AT_C and NF-AT_P reveals that they are products of distinct
10 genes with 73% amino acid identity in the Rel similarity region (Fig. 4), however, there is very little similarity outside this region. A murine cDNA for NF-AT_C was isolated and the predicted protein was found to be 87% identical to human NF-AT_C, and distinctly different from murine NF-AT_P.

15 EXAMPLE 1. Determination of the nucleotide and amino acid sequence of human NF-AT_C cDNA

This example represents the isolation and purification of this novel human NF-AT protein, NF-AT_C, the determination of the amino acid sequence of its fragments and the isolation and
20 sequencing of the cDNA clone encoding this protein.

The protein was purified from bovine thymus glands obtained from newborn calves. Approximately 20 bovine thymuses were homogenized to make a cytosolic extract which was then subjected sequentially to 1) ammonium sulfate precipitation, 2)
25 sulphopropyl Sepharose chromatography, 3) heparin agarose chromatography, 4) affinity chromatography using a multimerized binding site for NF-AT, with the sequence 5'-ACGCCCCAAGAGGAAAATTTGTTTCATACA-3' coupled to sepharose CL4B, and 5) HPLC on a reverse phase C4 column. The resulting
30 purified protein was subjected to cleavage with LysC/ArgC and fragments isolated by HPLC. The sequences of these individual fragments were then determined by automated Edman degradation. Sequences obtained included: LRNSDIELRKGETDIGR and LRNADIELR. Degenerate oligos corresponding to GETDIG (reverse primer) and
35 RNADIE (forward primer) were made. The degenerate oligo PCR primers had the following sequences:

A forward: (A/C)GIAA(C/T)GCIGA(C/T)AT(A/C/T)GA(A/G)

A reverse: ICC(A/G/T)AT(A/G)TCIGT(C/T)TCICC

To isolate the cDNA, oligonucleotide probes were made corresponding to the determined amino acid sequence and used as PCR primers to isolate a 45 base fragment from bovine cDNA prepared from the bovine thymus. The bovine PCR product

5 comprised the nucleotide sequence CTG CGG AAA which encodes -L-R-K-. The same 45 bp fragment can be amplified from human and mouse sources.

This bovine PCR product was then used to screen a cDNA library of the human Jurkat T cell line. Clones were
10 isolated at frequencies of about 1 in 100,000 to 1 in 200,000. A total of five human cDNA clones of various lengths were isolated. Two overlapping clones, one containing the 5' end and one containing the 3' end were ligated together using a unique EcoRI restriction site present in each clone, to produce
15 a full-length cDNA which corresponded in length to the messenger RNA determined by Northern blotting.

The sequence of the NF-AT_c cDNA was determined by the Sanger method and the complete nucleotide and predicted amino acid sequence is shown in Fig. 1. The initiator methionine
20 indicated in Fig. 1 was determined by fusing this reading frame to a glutathione transferase gene and transfecting the resultant clone into bacteria. The resultant clone produced a fusion protein of the proper molecular weight, indicating that the reading frame designated with the initiator methionine is
25 indeed the correct reading frame. The position of the stop codon was determined by a similar procedure. In addition, the stop codon corresponds to the reading frame for nine of the determined amino acid sequences.

The total NF-AT_c protein structure was aligned
30 against individual Rel proteins using a MacIntosh shareware program called DOTALIGN utilizing the alignment parameters of the FASTA programs. Significant homology was observed that corresponded to the Rel domains of these proteins. Enhanced amino acid residue alignment was done using ALIGN from the same
35 suite of programs. Alignment of the Rel similarity regions of NF-AT_c and NF-AT_p was done by hand with no insertions necessary, The Miyata alphabet (Miyata et al. (1979) J. Mol. Evol. 12: 214-236) was used to determine similar residues.

Fig. 4 shows results of such sequence alignments.

EXAMPLE 2. Expression of NF-AT_c in T and non-T cells

The cDNA shown in Fig.1 was fused to the *Hemophilus*
5 influenza hemagglutinin (HA) 12 amino acid epitope tag in the
determined reading frame and operably linked to the SR α
promoter in the vector pBJ5 (Lin et al, 1990, Science 249:677-
679). The resultant construct was transiently transfected by
electroporation into Jurkat human T lymphocytes, and into Cos
10 fibroblast cells. Expression of the epitope-tagged NF-AT_c
protein was determined by Western blotting of whole cell
extracts prepared from the transfected cells, using an antibody
(12CA5, Berkeley Antibody Co., CA) that detects the HA epitope.

Fig. 2 shows that NF-AT_c cDNA construct is able to express
15 a protein of approximately 120 kDA corresponding precisely in
size to that of the purified protein, in both Jurkat T cells
and Cos cells (see lanes 3 and 6 labeled NF-AT*. Lane 2 shows
as control, NF-AT without the epitope tag which cannot be
detected in the Western blot).

20

EXAMPLE 3. Transfection of NF-AT_c activates transcription in
both Cos and Jurkat cells

The NF-AT_c cDNA was operably linked to a portion of the
SV40 early gene promoter and the HIV transcription regulatory
25 regions in the pBJ vector. This expression vector was co-
transfected into Jurkat and Cos cells with either a) three
copies of NF-AT binding site linked to and directing
transcription of luciferase (results shown in Fig. 3A and 3B)
the entire IL-2 enhancer/promoter directing transcription of
30 luciferase (results shown in Fig. 3B). Cytosolic extracts were
prepared and luciferase assays carried out by standard
procedures (de Wet et al, 1987, Mol. Cell. Biol. 7:724-837).

The results demonstrate that in both Cos cells and Jurkat
cells, overexpression of the NF-AT_c protein dramatically
35 enhances NF-AT-dependent transcription by 50-1000 fold (see
Fig. 3A). In addition, overexpression of the NF-AT_c protein in
Cos cells activates the IL-2 promoter, which in the absence of
NF-AT_c cannot otherwise be activated (see Fig. 3B).

These results indicate that the cDNA clone encodes a functional NF-AT_c protein and that NF-AT_c is the protein which restricts expression of interleukin-2 to T cells.

5 EXAMPLE 4. NF-AT_c mRNA and Protein Expression

NF-AT_c mRNA is absent in Hela cells (Fig. 5, panel a, lane 7), a cell line incapable of IL-2 or NF-AT-dependent transcription, but is inducible in Jurkat cells (Fig. 5, panel a). This induction is sensitive to cyclosporin A, (CsA),
10 indicating that NF-AT_c may participate in an auto-stimulatory loop as CsA has been shown to block its nuclear association (Flanagan et al. (1991) Nature 352: 803-807). Two B cell lines, muscle tissue, Hep G2 cells and myeloid leukemia cells do not express NF-AT_c mRNA (Fig. 5, panel b). These
15 observations are consistent with the observed T cell-restricted pattern of IL-2 transcription and NF-AT activity. Previous studies (Verweij et al. (1990) J. Biol. Chem 265: 15788-15795) revealed NF-AT-dependent transcription predominantly in spleen, thymus and skin of transgenic mice expressing an NF-AT-
20 dependent reporter gene. Consistent with these observations, murine NF-AT_c mRNA shows the same pattern of expression (Fig. 5 panel c). Small amounts of NF-AT_c expression are seen in lung and heart, however, this may be due to contamination with circulating T cells. Murine NF-AT_p mRNA, also assayed by
25 quantitative ribonuclease protection, was found to be expressed at approximately equal levels in brain, heart, thymus and spleen (Fig 5, panel c). In contrast to NF-AT_c, NF-AT_p was not inducible by PMA and ionomycin (Fig 5, panel c).

METHODS. Specific human or mouse NF-AT_c or mouse NF-
30 AT_p cDNA fragments were used as templates for the synthesis of RNA transcripts. Ribonuclease protection was done according to Melton et al. (1984) Nucl. Acids. Res. 12: 7035-7056) using 10 µg of total RNA. Splenocytes and thymocytes were isolated and treated as described (Verweij et al. (1990) J. Biol. Chem 265:
35 15788-15795) before isolating RNA, otherwise whole tissue was used.

EXAMPLE 5. Functional Expression of NF-AT_c

NF-AT luciferase and IL-2 luciferase have been described (Northrop et al. (1993) J. Biol. Chem. 268: 2917-2923). β 28 luciferase was constructed by inserting a trimerized HNF-I recognition site (β 28) in place of the NF-AT recognition sites in NF-AT luciferase. The plasmid pSV2CAT (Gorman et al. (1982) Mol. Cell. Biol. 2: 1044-1050) was used as an internal control for transfection efficiency. Cells were transfected with 1.5 ug of luciferase reporter and 3 ug of expression construct as described. After 20 hours of growth, cells were stimulated for 8 hrs. with 20 ng/ml PMA plus or minus 2 uM ionomycin, and harvested for luciferase (de Wet et al. (1987) Mol. Cell. Biol. 7: 725-737) and CAT assays (Gorman et al. (1982) Mol. Cell. Biol. 2: 1044-1050).

Cos cells were transfected with epitope tagged NF-AT_c as described. Cos cells, Jurkat cells, and murine thymocytes were stimulated for 3hr. with PMA and ionomycin, Hela cells were stimulated for 3hr with PMA alone and nuclear extracts prepared as described (Fiebing et al. (1990) Genes & Dev. 4: 1823-1834). Cytosols were prepared from non-stimulated Cos cells. Gel mobility shifts were performed as previously described (Flanagan et al. (1991) Nature 352: 803-807; Northrop et al. (1993) J. Biol. Chem. 268: 2917-2923). Antisera were raised in mice immunized with bacterially expressed glutathione S-transferase fusion proteins using the vector pGEX-3X (Pharmacia) and purified on glutathione agarose. Fusion proteins contained NF-AT_c residues 12 to 143 (immune-1) and 12 to 699 (immune-2).

NF-AT_c, expressed in non T cell lines specifically activated transcription from the NF-AT site and the IL-2 promoter, (Fig. 6 panel a (left), and Fig. 6 panel b). In transiently transfected Jurkat cells, overexpression of NF-AT_c activated an NF-AT-dependent promoter but not an HNF-1-dependent promoter (Fig. 6 panel a (right)) or an AP-1 - dependent promoter. Transfection of the NF-AT_c cDNA gives rise to DNA binding activity that is indistinguishable from endogenous NF-AT (Fig. 6 panel c, lanes 1-4). Antibody directed against the HA epitope encoded by the transfected cDNA

induces a supershift of the NF-AT complex indicating that NF-AT_c participates in this activity. The nuclear NF-AT activity in transfected Cos cells comigrates with, and has the same binding specificity as, the native nuclear complex in T-cells (Fig. 6 panel c, lanes 4-11). Cytosolic extracts from NF-AT_c, transfected Cos cells can reconstitute NF-AT DNA binding activity when mixed with Hela nuclear extract (Fig. 6 panel c, lanes 12-16) as do cytosolic extracts from T-cells (Flanagan et al. (1991) Nature 352: 803-807; Northrop et al. (1993) J. Biol. Chem. 268: 2917-2923). Antisera raised against bacterially expressed fragments of NF-AT_c that have no similarity to NF-AT_p are able to induce a supershift of the endogenous NF-AT complex, but not the AP-1 complex, from Jurkat cells or thymocytes (immune-1 and immune-2 respectively, Fig. 6 panel d). Immune-2 antisera reduced the DNA-protein complex produced using murine thymic nuclear extracts significantly, consistent with the relatively equal representation of NF-AT_c and NF-AT_p peptides in the purified protein from bovine thymus.

EXAMPLE 6. NF-AT_c dominant negative mutant assayed in transient transfection assays

A dominant negative NF-AT_c, prepared after extensive deletion analysis of the cDNA, indicated that the amino terminal domain would block NF-AT-dependent function without affecting binding. This region of the cDNA is not found in NF-AT_p and hence can be used to assess the contribution of NF-AT_c to the activation of the IL-2 gene. The dominant negative NF-AT_c used consists of a carboxy terminal truncation of the epitope tagged NF-AT_c expression plasmid (*supra*) extending to the PvuII site at amino acid 463. Transfection of this dominant negative resulted in more than 90% inhibition of IL-2 promoter function as well as transcription directed by the NF-AT site (Fig 7). This effect was highly specific since transcription directed by the AP-1 site or the RSV promoter and enhancer were relatively unaffected (Fig 7). These results strongly indicate that NF-AT_c contributes substantially to IL-2 gene expression in T cells.

Dominant-negative NF-AT_c polypeptides or peptidomimetics thereof can be used as pharmaceutical antagonists of NF-AT-mediated activation of T cells. In one variation, such drugs can be used as commercial research reagents for laboratory testing and analysis of T cell activation and the like, among many other uses (e.g., immunosuppressant).

EXAMPLE 7. Post-Translational Modification of NF-AT_c

Post-translational modification of NF-AT_c was investigated in cells treated with agents that activate PKC or increase intracellular Ca⁺⁺. Cells were transfected with NF-AT_c as described in Fig. 2 and stimulated as shown for 2 hrs plus or minus 100ng/ml CsA. Whole cell lysates were analyzed by western blotting as in Fig. 2. The bulk of NF-AT_c in cells treated with ionomycin migrates faster than that in non-treated cells and this mobility shift is inhibited by CsA (Fig. 8, lanes 1, 3-4). This is consistent with a dephosphorylation event, possibly by direct action of calcineurin (Clipstone and Crabtree (1992) Nature 357: 695-697), however, any of a large number of processes could produce the observed mobility changes. There is evidence that NF-AT_p is a substrate for calcineurin, however, the mobility shifts produced by phosphatase treatment of NF-AT_p or NF-AT_c are far greater than those observed in Figure 8. These observations indicate that NF-AT_c is not a direct substrate of calcineurin. PMA treatment produces a slower migrating NF-AT_c (Fig. 8, lane 2); therefore, PKC-activated pathways likely contribute to NF-AT activity by modification of NF-AT_c in addition to activation of the nuclear component.

Although the present invention has been described in some detail by way of illustration for purposes of clarity of understanding, it will be apparent that certain changes and modifications may be practiced within the scope of the claims.

CLAIMS

1. An isolated NF-AT_c polypeptide comprising a polypeptide sequence of at least 25 consecutive amino acids which is substantially identical to a polypeptide sequence shown in Fig. 1.
2. An isolated NF-AT_c polypeptide of claim 1 which is substantially identical to the full-length NF-AT_c polypeptide sequence shown in Fig. 1.
3. An isolated NF-AT_c polypeptide of claim 1 which is a naturally-occurring polypeptide sequence in mammals.
4. An isolated NF-AT_c polypeptide of claim 3 which is a human NF-AT_c polypeptide.
5. An isolated NF-AT_c polypeptide of claim 4, which comprises at least two of the amino acid sequences shown in Table II.
6. An isolated NF-AT_c polypeptide of claim 1, which comprises amino acid 418 to amino acid 710 of the NF-AT_c polypeptide sequence of Fig. 1.
7. A polypeptide comprising the amino acids 1-418 of Fig. 1 and lacking an amino acid sequence consisting essentially of amino acids 419-716 of Fig. 1.
8. An isolated NF-AT_c polypeptide of claim 1 which is less than 716 amino acids long.
9. An isolated polynucleotide encoding a NF-AT_c polypeptide of claim 1.
10. An isolated polynucleotide of claim 9, wherein the NF-AT_c polypeptide comprises at least one of the following peptide sequences:

-NAIFLTVSREHERVGC-;

-AKTDRDLCKPNSLVVEIPFFRN-;

-LHGYLENEPLMLQLFIGT-; -EVQPKSHHRAHYETEGSR-;
 -PSTSPRASVTEESWL-; -SPRVSVTDDSWLGNT-;
 -GPAPRAGGTMKSAEEHYG-; -SHHRAHYETEGSRGAV-;
 -ASAGGHPIVQ-; -LRNSDIELRKGETDIGR-;
 5 -NTRVRLVFRV-; -TSLQVASNPIEC-.

11. An isolated polynucleotide of claim 10, wherein the NF-AT_c polypeptide comprises the sequence:

MPSTSFPVPSKFPLGPAAAVFGRGETLGPAPRAGGTMKSAEEHYGYASSNVSPALPLPTAHS
 10 TLPAPCHNLQTSTPGIIPPADHPSGYGAALDGCAGYFLSSGHTRPDGAPALESPRIETSCL
 GLYHNNNQFFHDVEVEDVLPSSKRSPSTATLSLPSLEAYRDPSCLSPASSLSSRSCNSEASSY
 ESNYSYPYASPQTSWPQSPCVSPKTTDPEEGFPRGLGACTLLGSPQHSPSTSPRASVTEESWL
 GARSSRPASPCNKRKYSLNGRQPPYSPHHSPTSPHGSPPRVSVTDDSWLGNTTQYTSSAIVAA
 INALTDDSSLDLGDGVPVKSRTTLEQPPSVALKVEPVGEDLGSPPPPADFAPEDYSSFQHIR
 15 KGGFCDQYLAVPQHPYQWAKPKPLSPTSMSPTLPALDWQLPSHSGPYELRIEVQPKSHHRAH
 YETEGSRGAVKASAGGHPIVQLHGYLENEPLMLQLFIGTADDRLLRPHAFYQVHRITGKTVST
 TSHEAILSNTKVLEIPLLPENSMRAVIDCACILKLNSDIELRKGETDIGRKNTRVRLVFRVH
 VPQPSGRTLQVASNPIECSQSAQELPLVEKQSTDSYPVVGKKMVLSGHNFLQDSKVIFV
 EKAPDGHVWEMEAKTDRDLCKPNSLVVEIPPFRNQRTSPVHVSFYVCNGKRKRSQYQRFYV
 20 LPANGNAIFLTVSREHERVGCFF

12. An isolated polynucleotide of claim 9, wherein the NF-AT_c polypeptide comprises the sequence from amino acid 419-716 of Fig. 1.

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13. An isolated polynucleotide comprising at least one of the following NF-AT_c polynucleotide sequences:

5'-TTC CTC CGG GGC GCG CGT GAG CCC GGG GCG AGG-3';
 5'-CAG CGC GGG GCG GCC ACT TCT CCT GTG CCT CCG CCC GCT GCT-3';
 30 5'-GCC GCG CGG ATG CCA AGC ACC AGC TTT CCA GTC CCT TCC AAG-3';
 5'-CCA ACG TCA GCC CCG CCC TGC CGC TCC CCA CGG CGC ACT CCA-3';
 5'-TTC AGA CCT CCA CAC CGG GCA TCA TCC CGC CGG CGG-3';
 5'-GCC ACA CCA GGC CTG ATG GGG CCC CTG CCC TGG AGA GTC CTC-3';
 5'-AGT CTG CCC AGC CTG GAG GCC TAC AGA GAC CCC TCG TGC CTG-3';
 35 5'-GTG TCT CCC AAG ACC ACG GAC CCC GAG GAG GGC TTT CCC-3';
 5'-AGC TGG CTG GGT GCC CGC TCC TCC AGA CCC GCG TCC CCT TGC-3';
 5'-TAC AGC CTC AAC GGC CGG CAG CCG CCC TAC TCA CCC CAC CAC-3';
 5'-GAC CAC CGA CAG CAG CCT GGA CCT GGG AGA TGG CGT CCC TGT-3';

5'-CCT GGG CAG CCC CCC GCC CCC GGC CGA CTT CGC GCC CGA AGA-3';
5'-GCT CCC CTA CCA GTG GCG AAG CCC AAG CCC CTG TCC CCT ACG-3';
5'-CTT CGG ATT GAG GTG CAG CCC AAG TCC CAC CAC CGA GCC CAC-3';
5'-CAT GGC TAC TTG GAG AAT GAG CCG CTG ATG CTG CAG CTT TTC-3';
5 5'-AAG ACC GTG TCC ACC ACC AGC CAC GAG GCT ATC CTC TCC AAC-3';
5'-TCA GCT CAG GAG CTG CCT CTG GTG GAG AAG CAG AGC ACG GAC-3';
5'-AAC GCC ATC TTT CTA ACC GTA AGC CGT GAA CAT GAG CGC G-3';
5'-AGA AAC GAC GTC GCC GTA AAG CAG CGT GGC GTG TGG CA-3'; and
5'-GCA TAC TCA GAT AGT CAC GGT TAT TTT GCT TCT TGC GAA TG-3'.

10

14. An isolated NF-AT_c polynucleotide of claim 13 which encodes a NF-AT_c polypeptide.

15

15. A host cell containing a NF-AT_c polynucleotide of claim 9.

20

16. A host cell of claim 15, wherein said host cell expresses the NF-AT_c polynucleotide thereby producing a NF-AT_c polypeptide.

25

17. A host cell of claim 15 further comprising an NF-AT-dependent reporter gene.

18. A composition comprising a substantially purified NF-AT_c polypeptide which is substantially identical to a polypeptide sequence shown in Fig. 1.

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810	830	850	870	890	
234	Y E S N Y S Y P Y A S P Q T S P N Q S P C V S P K T T D P E E G F	266			
910	930	950	970	990	
267	P R G L G A C T L L G S P Q H S P S T S P R A S V T E E S W L G A R	300			
1010	1030	1050	1070	1090	
301	S S R P A S P C N K R K Y S L N G R Q P P Y S P H S P T P S P H	333			
1110	1130	1150	1170	1190	
334	G S P R V S V T D D S W L G N T T Q Y T S S A I V A A I N A L T T	366			
1210	1230	1250	1270	1290	
367	D S S L D L G D G V P V K S R K T T L E Q P P S V A L K V E P V G E	400			
1310	1330	1350	1370	1390	
401	D L G S P P P A D F A P E D Y S S F Q H I R K G G F C D Q Y L A	433			
1410	1430	1450	1470	1490	
434	V P Q H P Y Q W A K P K P L S P T S T N S P T L P A L D W Q L P S	466			
1510	1530	1550	1570	1590	
467	H S G P Y E L R I E V Q P K S H H R A H Y E T E G S R G A V K A S A	500			

FIG. 1A-2.

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501	ceggaggacccccatcgtagctgaatgagccgctgactgctgagcttttcaittggagcgcgagcagccgctgctgcgc	1610	1650	1670	1690	533
	<u>G G H P I V Q L H G Y L E N E P L M L Q L F I G T A D R L L R P</u>					
534	gacagcctttacacggtagcacggagacggtagtcacccacgacgaggtactctctcaccacccaaagtcctggagatccac	1710	1750	1770	1790	566
	<u>H A F Y Q V H R I T G K T V S T T S H E A I L S N T K V L E I P L</u>					
567	ctgcaggagacacgcatggagcgctcatfigactgtgcggaaactcgaaactcgaaactcgaaactcgaaactcgaaactcg	1810	1850	1870	1890	600
	<u>L P E N S M R A V I D C A G I L K L R N S D I E L R K G E T D I G R</u>					
601	gagagacacacggtagcgctggtttccgcttcacgtccgcacacacgagcgcgctgctgcaggtggctcaccacacacacac	1910	1950	1970	1990	633
	<u>K N T R V R L V F R V H V P Q P S G R T L S L Q V A S N P I E C S</u>					
634	ccagcgctcagctcaggagctgcctctggtggagacgagacgacgactatccggtcgtggcgagagagagatggtcctgtctggcacaac	2010	2050	2070	2090	666
	<u>Q R S A Q E L P L V E K Q S T D S Y P V V G G K K M V L S G H N F</u>					
667	ctgcaggacacacgctcatcttctgtagagaaagccccagatggccacatgtctggagatgagcgaaacatgacgggacctgtgcagccgaatt	2110	2150	2170	2190	700
	<u>L Q D S K V I F V E K A P D G H H V W E M E A K T D R D L C K P N S</u>					
701	ctctggtggttagatccgcgcatttcggaaatcaggaggataaccagccccgttcaagctcagtttctacgtctgcaacgggagagagagagagag	2210	2250	2270	2290	733
	<u>L V V E I P P F R N Q R I T S P V H V S F Y V C N G K R K R S Q Y</u>					
734	ccagcgctttcacctacattccgcacacgggtaacgcatctttctaacgttaacgctgaacatgagcgctgggtgccttttctaacgagcagagac	2310	2330	2350	2370	
	<u>Q R F T Y L P A N G N A I F L T V S R E H E R V G C F F</u>					

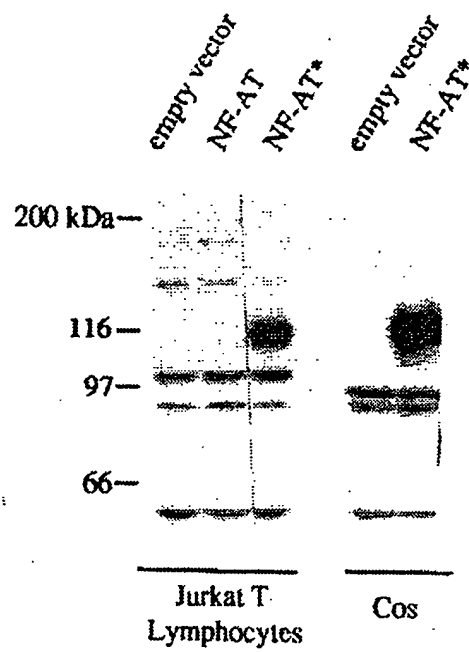
FIG. 1B-1.

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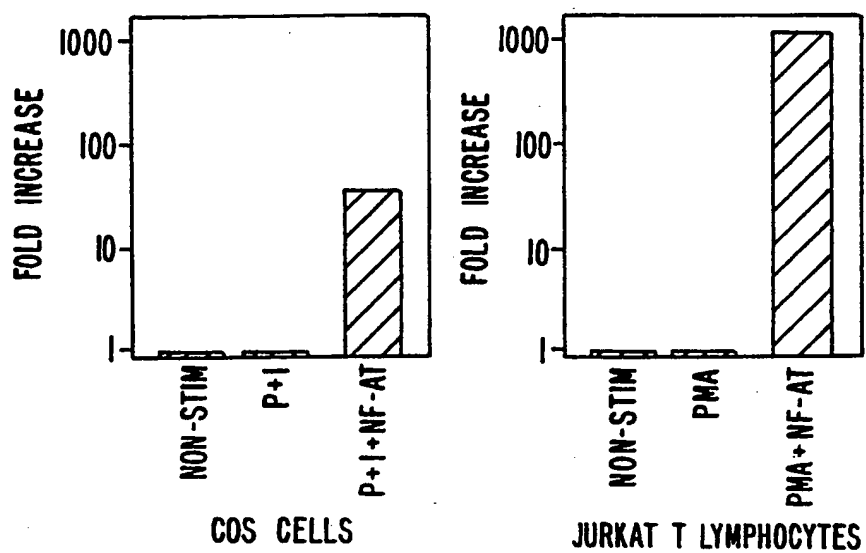
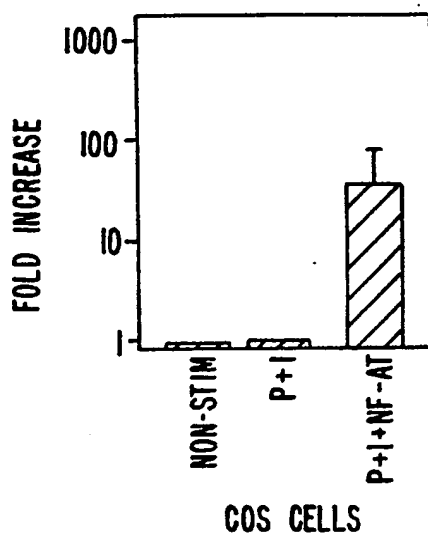
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2510	2530	2550	2570	2590
aagcttcggatgcatcttcccttgatccctgtgggggtgggggcgggggttgcatactcagatagtcaagggtattttgcttcttgcgaatgtatacca				
2610	2630	2650	2670	2690
gcccaggggaaaaaatggcctctctgctccaaaaaacctgaggggtactggtagtgcaatttgacccctaaagctgcttacgggtgaaagggcaaatagggtat				
2710	2730			
agctattttgcaggacaccttaggaataaacctttgcttttaaaaaaaa				

FIG. 1B - 2.

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**FIG. 2.**

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*FIG. 3A.**FIG. 3B.*

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MDORSAL	T K N V	K K P Y	V K I	T E	Q P A	K K	A L	R F F	Y Y	E	G E	R R	S S	A A	G S	P P	V N	S T	P E N	K T
C-REL	M A S	L Y N	P Y I	E I	Q P P	K K	A G	M F	R R	F F	C C	E G	R R	S S	A A	G S	P P	V N	S T	P E N
NFKB p50	I P L	S T D	G P Y	I Q E	Q P P	K K	A G	M F	R R	F F	C C	E G	R R	S S	A A	G S	P P	V N	S T	P E N
NFKB p65	P P A	Q A S	G P Y	V E L	Q P P	K K	A G	M F	R R	F F	C C	E G	R R	S S	A A	G S	P P	V N	S T	P E N
NFATc	Q L P	S H S	G P Y	V E L	Q P P	K K	A G	M F	R R	F F	C C	E G	R R	S S	A A	G S	P P	V N	S T	P E N
NFATp	P L S	N Q S	G P Y	V E L	Q P P	K K	A G	M F	R R	F F	C C	E G	R R	S S	A A	G S	P P	V N	S T	P E N
MDORSAL	Y P T	T T E	V G Y	K G R	A V	V V	S C V	T K D	P Y R	P	H H	N L	V G	K E	G C	R	K	G	V C	T L
C-REL	Y P T	T T E	V G Y	K G R	A V	V V	S C V	T K D	P Y R	P	H H	N L	V G	K E	G C	R	K	G	V C	T L
NFKB p50	Y P T	T T E	V G Y	K G R	A V	V V	S C V	T K D	P Y R	P	H H	N L	V G	K E	G C	R	K	G	V C	T L
NFKB p65	Y P T	T T E	V G Y	K G R	A V	V V	S C V	T K D	P Y R	P	H H	N L	V G	K E	G C	R	K	G	V C	T L
NFATc	Y P T	T T E	V G Y	K G R	A V	V V	S C V	T K D	P Y R	P	H H	N L	V G	K E	G C	R	K	G	V C	T L
NFATp	Y P T	T T E	V G Y	K G R	A V	V V	S C V	T K D	P Y R	P	H H	N L	V G	K E	G C	R	K	G	V C	T L
MDORSAL	N S E	R R P	L V G	S N L	G I	Q C C	K K K	D E K	A A	E A	K A R	-	E E	I R	V V	F F	K T	G F	S H	R F
C-REL	N S E	R R P	L V G	S N L	G I	Q C C	K K K	D E K	A A	E A	K A R	-	E E	I R	V V	F F	K T	G F	S H	R F
NFKB p50	N S E	R R P	L V G	S N L	G I	Q C C	K K K	D E K	A A	E A	K A R	-	E E	I R	V V	F F	K T	G F	S H	R F
NFKB p65	N S E	R R P	L V G	S N L	G I	Q C C	K K K	D E K	A A	E A	K A R	-	E E	I R	V V	F F	K T	G F	S H	R F
NFATc	N S E	R R P	L V G	S N L	G I	Q C C	K K K	D E K	A A	E A	K A R	-	E E	I R	V V	F F	K T	G F	S H	R F
NFATp	N S E	R R P	L V G	S N L	G I	Q C C	K K K	D E K	A A	E A	K A R	-	E E	I R	V V	F F	K T	G F	S H	R F
MDORSAL	A Y L	Q A E	G G D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C-REL	A Y L	Q A E	G G D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NFKB p50	A Y L	Q A E	G G D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NFKB p65	A Y L	Q A E	G G D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NFATc	A Y L	Q A E	G G D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NFATp	A Y L	Q A E	G G D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

FIG. 4-1.

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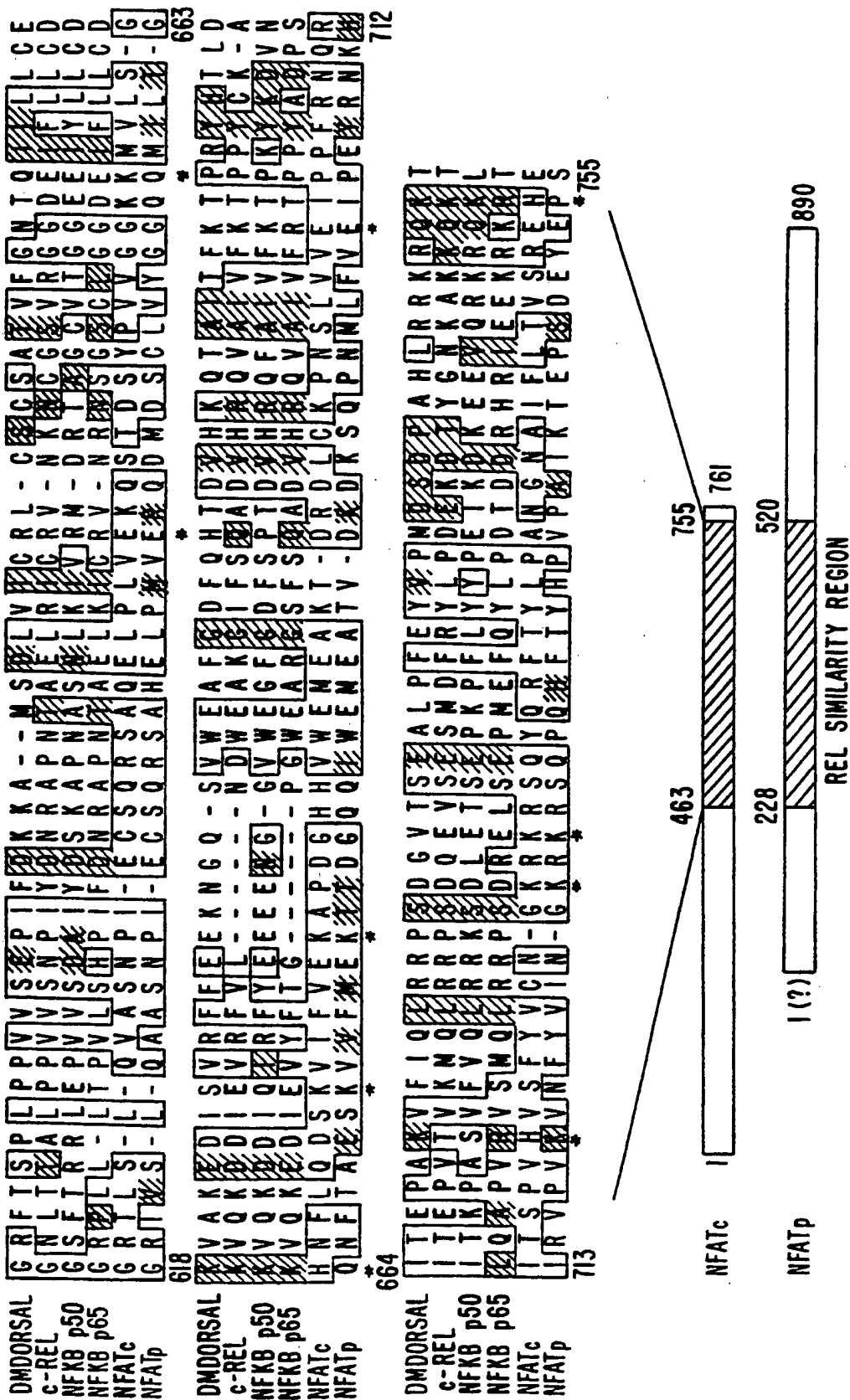


FIG. 4-2.

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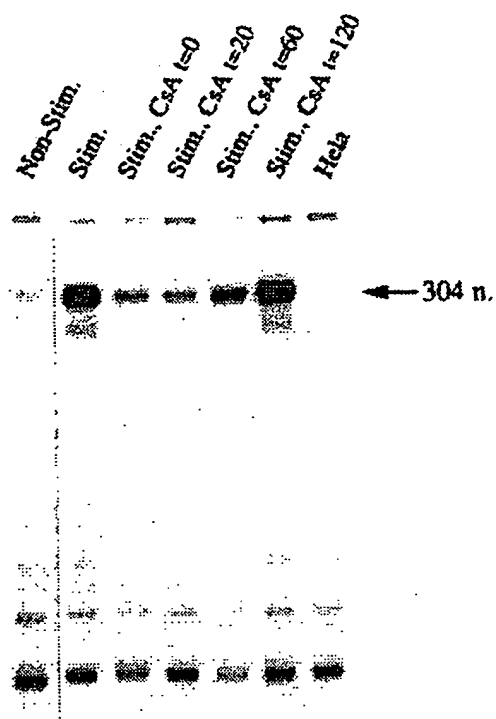


FIG. 5A.

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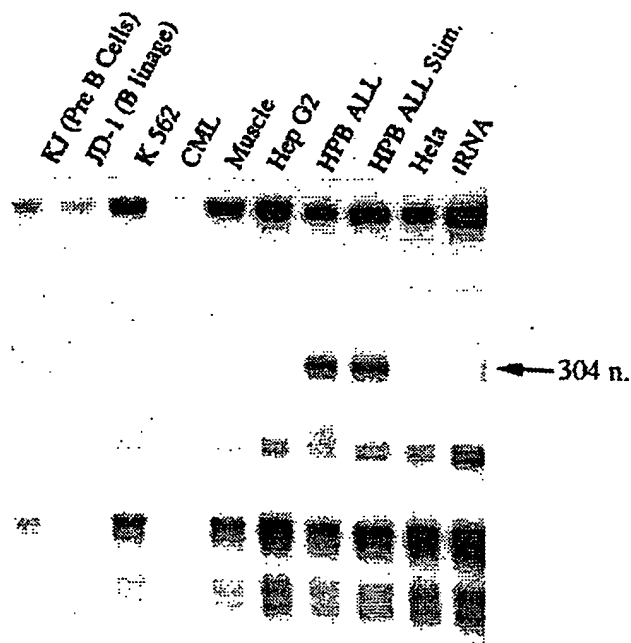


FIG. 5B.

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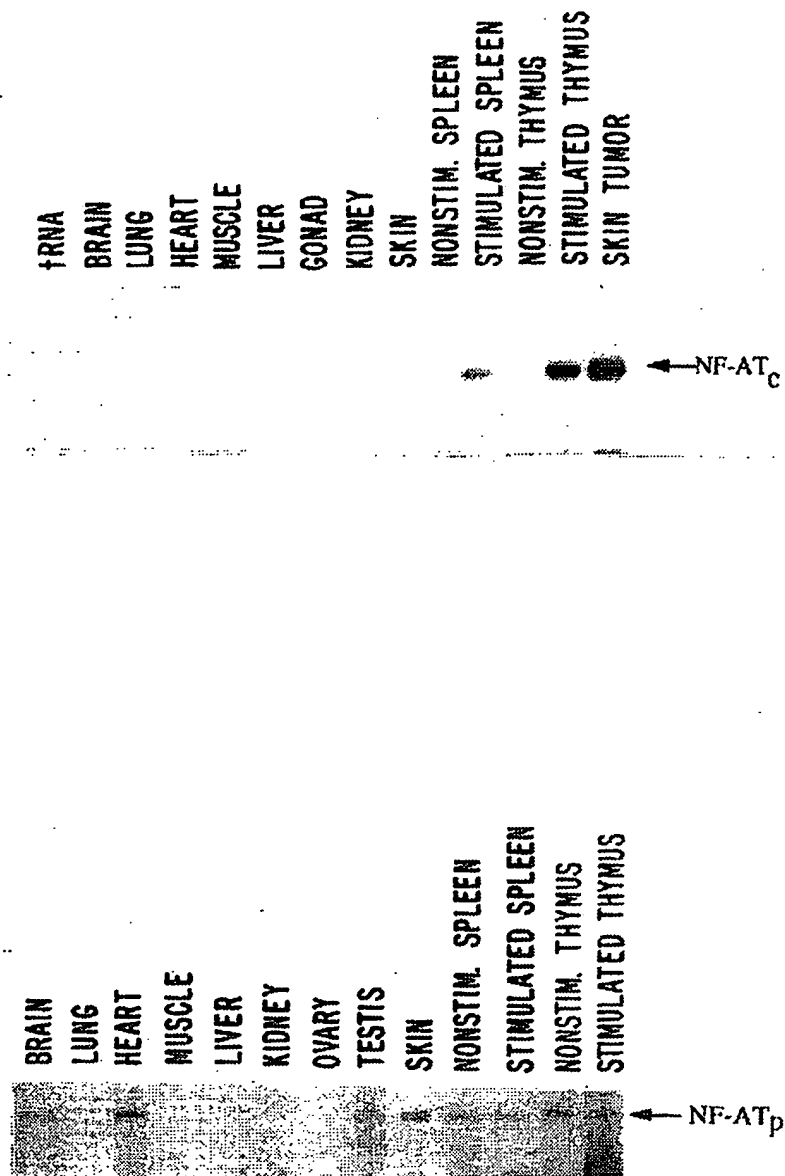


FIG. 5C.

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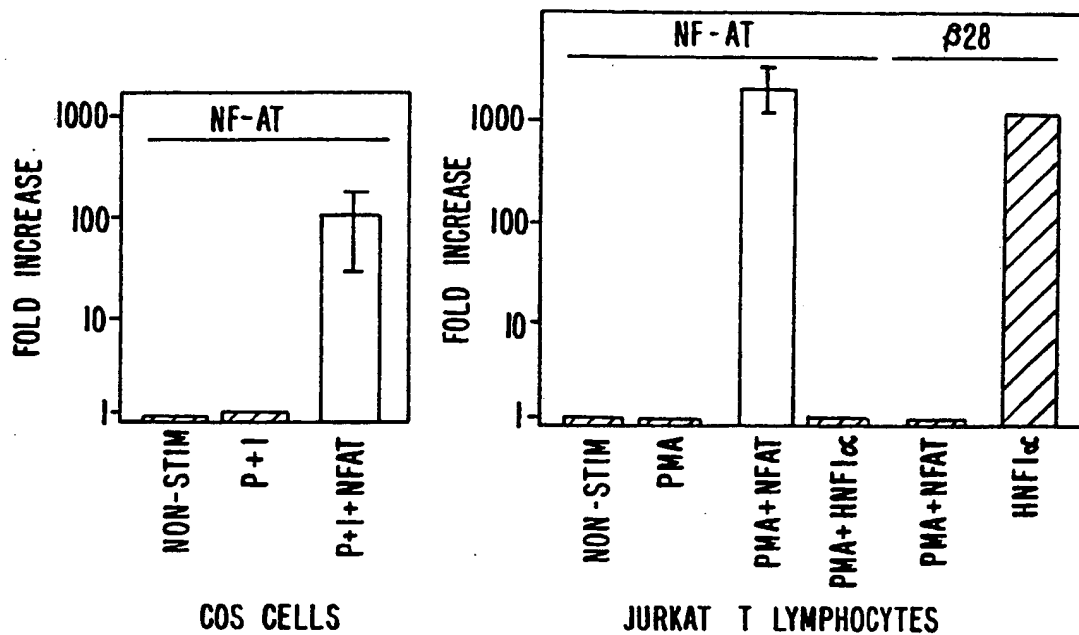


FIG. 6A.

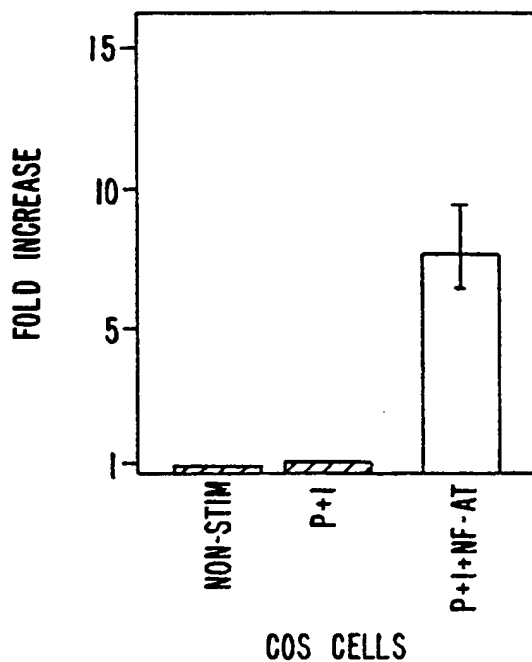


FIG. 6B.

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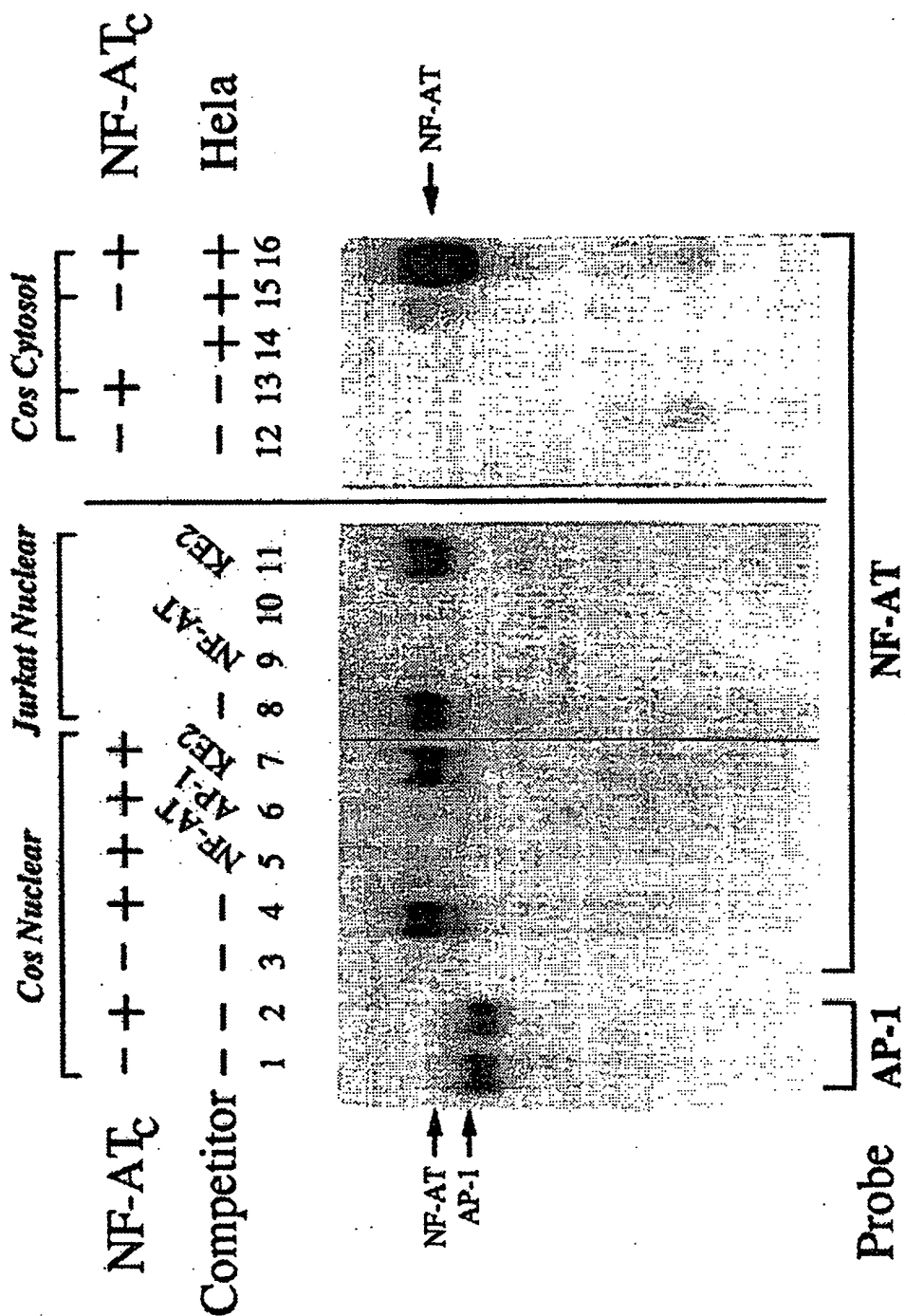
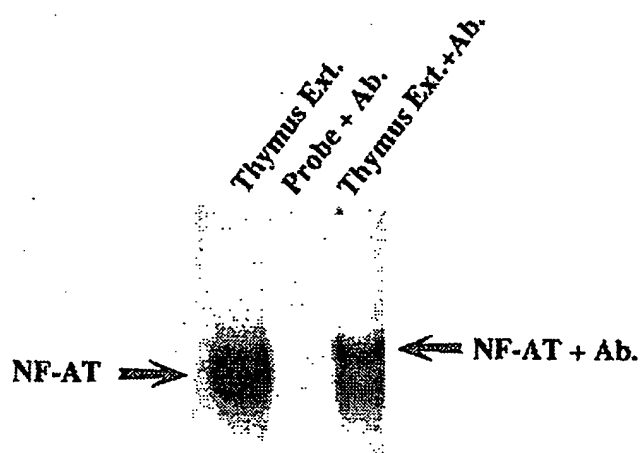
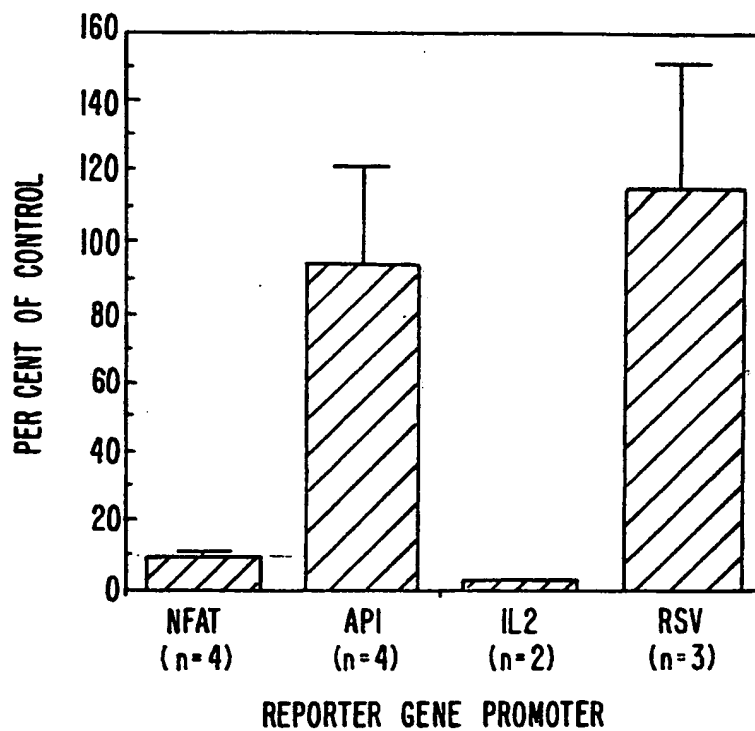


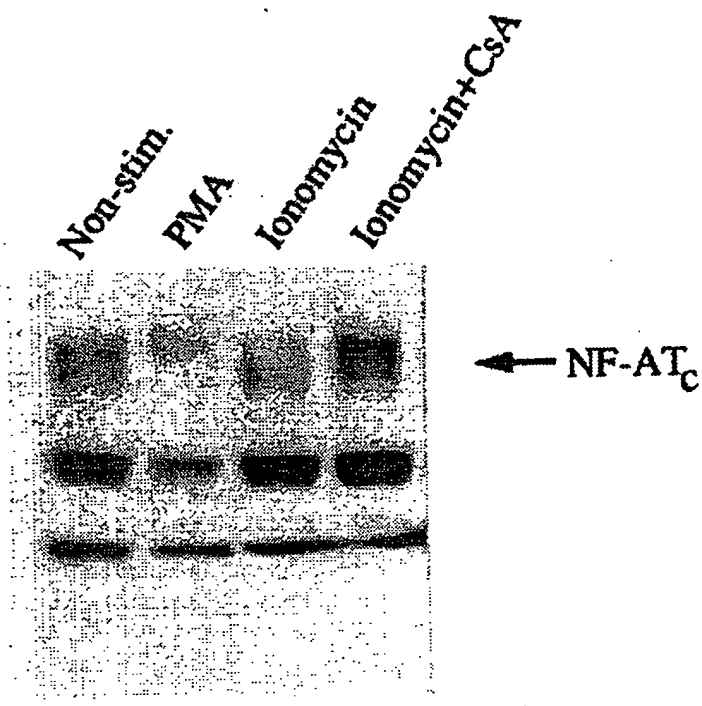
FIG. 6C.

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**FIG. 6D.**

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**FIG. 7.**

**FIG. 8.**

INTERNATIONAL SEARCH REPORT

 Intern. application No.
PCT/US94/10724
A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07H 17/00; C07K 1/00, 7/00, 14/00, 14/46; C12N 15/00

US CL :536/23.5; 435/252.3, 320.1; 530/350

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.5; 435/252.3, 320.1; 530/350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Dialog, medline, STN

search terms:NF-ATc, NF-AT, T cells, leucocyte, transcription, Figure 1

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Nature, Volume 357, issued 25 June 1992, N. A. Clipstone et al., "Identification of Calcineurin as a Key Signalling Enzyme in T-Lymphocyte Activation", pp. 695-697, see the entire document.	1-18
A	Nature, Volume 352, issued 29 August 1991, W. M. Flanagan et al., "Nuclear Association of a T-Cell Transcription Factor Blocked by FK-506 and Cyclosporin A", pp. 803-807, see entire document.	1-18
A	Nature, Volume 356, issued 30 April 1992, J. Jain et al., "Nuclear Factor of Activated T Cells Contains Fos and Jun", pp. 801-804, see entire document.	1-18

☒ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

29 DECEMBER 1994

Date of mailing of the international search report

13 JAN 1995

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INTERNATIONAL SEARCH REPORT

Internat application No.
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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Proc. Natl. Acad. Sci. USA, Volume 87, issued December 1990, B. E. Bierer et al., "Two Distinct Signal Transmission Pathways in T Lymphocytes are Inhibited by Complexes Formed Between an Immunophilin and either FK506 or Rapamycin", pp. 9231-9235, see entire document.	1-18
P, A	Nature, Volume 369, issued 09 June 1994, Alain Israel, "NF-AT Comes Under Control", pp. 443-444, see entire document.	1-18